NEUTROPHILS IN BACTERIAL PNEUMONIA; INFLUX AND CLEARANCE

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PhD The University of Edinburgh 1997

Declaration

The preparation of this thesis and experiments described within are the work of Doctor Roderick A. Lawson, except as specifically acknowledged in the text. In particular, Ms. Helen Caldwell, Department of Pathology, University of Edinburgh Medical, kindly prepared specimens for histological examination.

Acknowledgements

I would very much like to acknowledge the help and support of a number of people. My supervisor, Prof. C. Haslett, made this thesis possible, and his help, encouragement and patience whilst the work was undertaken is greatly appreciated. My deputy supervisor, Dr. W. MacNee, provided helpful technical suggestions. Dr. I. Dransfield and Dr. E. Chilvers contributed a lot of time in knowledgeable discussion, as did other members of the Rayne Laboratory where the work took place. Dr. S. Hannah heroically attempted to reduce my errors by proof reading; any errors remaining are entirely my responsibility.

I would also like to express my heartfelt thanks to my wife, Karen, who has had a lot to put up with.

Abstract

Despite the advent of powerful modern antibiotics, pneumonia continues to be of great importance. Although most cases of community acquired pneumonia (CAP) will recover fully, some (including those previously fit and well) may die. Many hospital patients will suffer nosocomial pneumonia (NP), with a high mortality rate. This is particularly true on the intensive care unit.

The neutrophil granulocyte is of particular importance in the defence of the lung. It contains many substances that are bactericidal. During pneumonia, it is recruited from the intravascular space into the lung interstitium and the air-spaces. There, its bactericidal products are capable not only of damaging and killing bacteria, but also of causing 'by-stander' damage to the host lung. During the course of pneumonia it is important that neutrophils are not only recruited rapidly to defend against bacteria, but that their recruitment should cease as soon as adequate numbers are attained or their function is no longer required. Not only that, but the neutrophils that have already been recruited must then be safely and speedily removed from the site of pneumonia.

To investigate these dynamic processes, a rabbit model of pneumonia was used. Bacteria were instilled to a localised area via a fibre-optic bronchoscope. This allowed strict definition of the time of onset of pneumonia, and study of subsequent evolving processes. Two organisms were used; 1) Streptococcus pneumoniae, an organism characteristic of CAP and; 2) Escherichia coli, an organism characteristic of NP. It was hypothesised that in pneumonia due to the former (PneuS), the usual remarkably complete recovery witnessed clinically is due to the tissue load of neutrophils being carefully controlled. By contrast, pneumonia due to the latter (pneuE) is clinically much more severe and lung-damaging, due to a larger lung neutrophil burden. This could be because of earlier cessation of neutrophil influx and/or more rapid neutrophil clearance in pneuS than pneuE.

Radiolabelled neutrophils from donor animals were injected at intervals after induction of pneumonia to assess the magnitude of ongoing neutrophil recruitment. In pneuS, neutrophil influx was significantly elevated above control levels at 6 hour but not 30 hour or subsequent time points. In pneuE, neutrophil influx was at least as high at 30 hours as at 6 hours. This confirms the hypothesis that neutrophil influx is more prolonged in pneuE than in pneuS.

The requirement for the CD18 adhesion molecule, (known to be important in recruitment of neutrophils) has been shown to change with time in the peritoneum. This could be an important facet of control of the development of inflammation. In pneuS, neutrophil recruitment is known to be CD18 independent. The previous finding of others that such antibodies inhibit neutrophil recruitment early (6 hours) in pneuE was confirmed. It was found there was no change in this CD18 dependency later on (at 30 hours).

The chemokine IL-8 is thought to be particularly important in the recruitment of neutrophils. An anti-IL-8 blocking antibody was used to assess the importance of IL-8 in neutrophil recruitment at 6 and 30 hours. Although this inhibited IL-8 induced neutrophil shape change *in vitro* and intradermal IL-8 induced neutrophil influx *in vivo,* iand reversed the prolonged retention of tracer neutrophils in pneumonic lungs minutes after injection, the antibody failed to block neutrophil influx in either type of pneumonia at 6 or 30 hours. Indeed, there was a trend towards increased influx after treatment. High dose antibody produced the same effect. These surprising results are partly explained by higher broncho-alveolar lavage (BAL) and plasma levels of IL-8 after antibody treatment. This may be due to IL-8/anti-IL-8 antibody complexes amplifying the inflammatory response, although no endothelially bound anti-IL-8 was detected. Alternatively, it may represent the release of negative feedback on IL-8 production.

In both pneuS and pneuE, the early peak in BAL neutrophil numbers was followed by an increase in the number of apoptotic neutrophils. This in turn was followed by an

increased number of alveolar macrophages containing apoptotic bodies. Trypan blue positive, necrotic neutrophils were rare. This is consistent with neutrophil apoptosis and associated macrophage phagocytosis, (a process that limits the release of toxic neutrophil products), playing an important role in neutrophil clearance in pneumonia.

BAL from animals with pneumonia promoted rabbit neutrophil apoptosis *in vitro*. In pneuS (but not pneuE) this correlated weakly though significantly with the amount of apoptotic neutrophils recovered from BAL and with BAL levels of IL-8, gro and MCP-1. It is suggested that a factor is elaborated within the lung during inflammation that promotes neutrophil apoptosis, thus giving negative feedback control on the lung neutrophil burden. The different relationships found between *in vitro* and *in vivo* in pneuS and pneuE may again contribute to the different clinical outcomes in the two diseases.

In summary, an animal model demonstrated that neutrophil influx was more prolonged in pneuE than in pneuS. It is suggested this contributes to the more severe manifestations of the former clinically. CD18 was important to the influx of neutrophils in pneuE late as well as early in the disease. Anti-IL-8 antibody failed to inhibit neutrophil influx in either type of pneumonia, which may be due to immune-complex formation or release of negative feedback controls. Evidence that neutrophil apoptosis is involved in their clearance during pneumonia was obtained, together with evidence that a factor is released to promote neutrophil apoptosis and thus provide negative feedback control of inflammation during pneumonia. The control may differ in PneuS and PneuE, affecting outcome.

Abbreviations

6.5e	Name of an anti-CD18 antibody
ANOVA	Analysis of variance
ARDS	Adult respiratory distress syndrome
ARIL.8.2	Name of an anti-IL-8 antibody
BAL	Broncho-alveolar lavage
BSA	Bovine serum albumen
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
C5a	Fifth fragment of complement
C5f	Complement fragments
CAP	Community acquired pneumonia
E. coli	Escherichia coli
EDTA	Ethylenediamenetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMA	Epithelial membrane antigen
FITC	Fluorescein isothiocyanate
FMLP	Formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
gro	gro/melanocyte growth stimulatory activity
H&E	Haematoxylin and eosin stain
HIV	Human immunodeficiency virus
HUVEC	Human umbilical vein endothelial cell

IFN-γ	Interferon γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
In-wbc	^{111}indium labelled neutrophils
LAI effect	Leukocyte adhesion inhibitory effect
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MCP-1	Macrophage chemotactic peptide-1
NSD	No significant difference
om	Original magnification
PBS	Phosphate buffered saline
pneuE	Pneumonia due to Escherichia coli
pneuS	Pneumonia due to Streptococcus pneumoniae
PMA	Phorbol myristate acetate
PPP	Platelet poor plasma
ROI	Region of interest
SEM	Standard error of the mean
Strep. pneumoniae	Streptococcus pneumoniae
TBS	Tris buffered saline
Tc-rbc	^{99m}technetium labelled erythrocytes
TNF α	Tumour necrosis factor α

1. INTRODUCTION

"Sir William Osler called pneumonia 'the Captain of the men of death'. Although progress has been made to reduce the rank from Captain, greater efforts are needed to improve our understanding of the pathogenesis, prevention, diagnosis, and treatment of this infection" (Craven and Steger, 1989)

1.1 Scope and aims of this thesis

This thesis examines neutrophil granulocyte recruitment to and clearance from the lung in the context of pneumonia. Normally the neutrophil is produced in the bone marrow, before release into the circulation, where it has a limited lifespan. In pneumonia (as in other forms of acute inflammation) the neutrophil leaves the circulation and enters the tissues to perform a valuable defensive function. Paradoxically its presence may also be associated with detrimental injury. Hence, the neutrophil's safe and efficient clearance from the tissues is also of prime importance.

The thesis begins by reviewing the continuing importance of pneumonia, and describes some of the important interactions between invading bacteria and the body's defences. It then describes the normal circulating pattern of the neutrophil, drawing attention to the unique relationship the neutrophil has with the pulmonary circulation. Following this, it describes how this alters in disease, and how the neutrophil is induced to pass from the circulation to the tissues, emphasising the rôles of adhesion molecules (particularly the β_2 integrins) and chemotactic factors (particularly interleukin-8) in this. It then goes on to describe evidence that neutrophil clearance is an actively controlled event in which apoptosis (programmed cell death) is of great importance.

Much of the current knowledge of this whole process is derived from important *in vitro* experiments which have provided great molecular and cellular insights. However, there is a need to re-integrate this information to understand how the various factors contribute

and interact *in vivo*. This thesis attempts particularly to examine data that addresses this need to re-integrate knowledge, giving particular prominence to *in vivo* models. In particular, it emphasises that acute inflammation is a dynamic and evolving process that has a beginning, a middle and an end, and is the result of a dynamic interaction between host and inciting stimulus. Inflammation is often regarded as a light with a switch that may be either on or off; but it may be much more appropriate to describe it as a range of lights controlled by an array of dimmers!

The thesis also highlights the fact that, because of its special relationship with the pulmonary microcirculation, the neutrophil may behave differently during pulmonary inflammation (as typified by pneumonia) compared to inflammation elsewhere.

Original work is then described and discussed. Clinical observation has suggested that some types of pneumonia are characteristically associated with more tissue damage than others. It was hypothesised that there is a greater tissue neutrophil burden in the latter than the former, contributing to damage. Neutrophil accumulation is the balance of influx and clearance so these processes were examined separately, something that has been rarely performed in the past.

In chapter one, the pattern of neutrophil influx in two representative types of pneumonia is examined, to establish whether there is a different pattern in each. In chapters two and three respectively, the functional contribution of two molecules, CD18 and IL-8, to neutrophil influx during the course of pneumonia is then examined. These molecules were selected on the basis of many previous studies suggesting a rôle in neutrophil influx. However, in both cases, there have been no previous studies to examine whether their contribution specifically to neutrophil influx varies with time. This question is addressed for each molecule in turn.

Increased tissue neutrophil burden and hence damage could result from less efficient clearance of neutrophils in some types of pneumonia. Chapter four looks specifically at

apoptosis, a process that could be important in clearing neutrophils from the inflamed lung. It is hypothesised that this is an actively controlled process, important to successful resolution of inflammation. Evidence for its existence and control during pneumonia is sought.

1.2 Pneumonia and its importance.

Pneumonia is infection of the lung, giving rise to inflammation of the air spaces. This changes the lung from its normal soft, sponge like character to a much more solid consistency, a process known as consolidation.

There is a perception among some that the importance of bacterial pneumonia has diminished with the development of modern antibiotics, which may be highly active against the causative organisms. There is no doubt that antibiotics have cut overall mortality significantly. A review of 34,883 patients with pneumococcal pneumonia between 1938 and 1941 found an untreated mortality rate of 30.5%. This was reduced to 14.9% with serum treatment, and 7.6% with the use of sulphonamides (Finland, 1979).

Despite such advances, pneumonia remains of great significance. Overall, it is the sixth leading cause of death and the highest cause of death due to infectious disease in the USA (Garibaldi, 1985, and U.S. Department of Commerce, Statistical Abstract of the United States, 1984). The subject is made more serious by the progressive development of microbial resistance to antibiotics (Campbell, 1995, Doern, 1995). Studies showing that the mortality rate for bacteraemic pneumococcal pneumonia remains high (Hook et al, 1983, Gruer, et al 1984) led to the pessimistic suggestion (Bates, 1989) that even modern intensive care merely prolongs death rather than saving lives in severe primary pneumonia, although more recent data suggests that this extreme view is not justified (The British Thoracic Society Research Committee and the Public Health Laboratory Service, 1992).

1.3 Classification of pneumonia

Pneumonia may be classified in a number of ways. Traditionally, a distinction has been made between lobar pneumonia and bronchopneumonia. In the former, the pneumonia is confined within a lobe of the lung, its spread being limited by fibrinous exudate. In the latter, areas of consolidation are more widespread, being found among a number of lobules along the course of one or more bronchi, due to spread of infection along the airways (Coalson, 1995). There is a degree of associated bronchitis and bronchiolitis.

Pneumonia due to the pneumococcus, Streptococcus pneumoniae tends to be associated with lobar pneumonia (Coalson, 1995). However, lobar pneumonia may be due to other organisms, and broncho-pneumonia may be due to the pneumococcus. Thus, this distinction has not been sufficient to be of value pathologically or clinically (Farr et al, 1989, MacFarlane et al, 1984, Sue, 1994).

A more useful distinction is that between pneumonia acquired outside the hospital, community acquired pneumonia (CAP), and that acquired inside the hospital, nosocomial pneumonia (NP). The spectrum of causative organisms and the prognosis differ significantly in the two groups (American Thoracic Society, 1993, American Thoracic Society, 1996, Research Committee of the British Thoracic Society and the Public Health Laboratory Service, 1987, The British Thoracic Society, Research Committee and the Public Health Laboratory Service, 1992, Campbell, 1994, Coalson, 1995, Faas, 1993, George, 1995, Griffin and Meduri, 1994, Meyer. and Finch, 1992, Niederman, 1994, Scheld, 1991).

1.4 Community acquired pneumonia

1.4.1 Incidence

It is estimated that, in the United States, there are 4 million cases of community acquired pneumonia (CAP) annually (Garibaldi, 1985), or 0.68 to 2.6 per 1000 population

(McKinsey and Bisno, 1988). In a prospective study of 4 027 lower respiratory tract infections in the community in Nottingham, UK, 5.6% were found to be pneumonia (Woodhead et al, 1987). Of these, about 15 % required hospital admission. Of patients admitted to hospital, 5 to 11 % require admission to the intensive care unit (Lévy et al, 1988, and Research Committee of the British Thoracic Society and the Public Health Laboratory Service, 1987).

1.4.2 Bacterial Aetiology

A prospective multicentre study of British hospitals examined 453 cases of CAP and identified a bacteriological cause in 67 %. Thirty-three % had infection due to Strep. pneumoniae, 18 % due to Mycoplasma pneumoniae, and 7% due to influenza A. In addition, many of the cases in which a pathogen was not formally identified were likely to be due to Strep. pneumoniae. Many patients had received an antibiotic to which this organism was sensitive prior to hospital admission. In patients not receiving antibiotics on admission who were tested for the presence of pneumococcal antigen, > 50 % were positive. Fifty % of patients for whom the aetiology of pneumonia was not detected using more routine methods such as those employed in the British Thoracic Society study had evidence of pneumococcal infection on more sensitive, invasive testing (Jimenez et al, 1994).

Studies in many parts of the world have confirmed Strep. pneumoniae as the most common pathogen in CAP, including Finland (Kauppinen et al 1995), the Netherlands (Bohte et al, 1995), Ethiopia (Aderaye, 1994), Cameroon (Koulla-Shiro et al, 1993) and Denmark (Ostegaard and Andersen, 1993). It has also been found to be the commonest cause of CAP in children (Bahl et al, 1995), the elderly (Woodhead, 1994), and populations with a high prevalence of HIV infection (Mundy et al, 1995). In addition it is the leading cause of severe community acquired pneumonia, being the leading cause of CAP necessitating admission to the intensive care unit (The British Thoracic Society Research

Committee and the Public Health Laboratory Service, 1992, Leroy et al, 1995). The occasional exceptions to the overwhelming importance of Strep. pneumoniae are likely to be due to unusual local conditions, e.g. the very high rates of tuberculosis in Singapore make this the commonest form of CAP requiring hospitalisation on the island (Hui et al, 1993).

1.4.3 Outcome

The total mortality rate for CAP is low. In a study examining all cases occurring in a defined community, the mortality rate was 3 %. Seventy-eight % were managed in the community, with only one death occurring in this group (Woodhead et al, 1987).

Although the overall mortality rate is low, CAP may be severe, especially in some subgroups. Of those hospitalised during Woodhead et al's study the mortality rate was 11.5 %, but patients aged 70-79 had a ten fold greater mortality rate than patients aged 20-29. Similar findings were made in a hospital survey of CAP (Research Committee of the British Thoracic Society and the Public Health Laboratory Service, 1987). Of the deaths that did occur in this study, 27 % occurred within 48 hours of admission to hospital, suggesting that this was due to rapid overwhelming disease.

In survivors, the mean length of hospital stay was 11 days. 6 weeks after the onset of pneumonia, 79 % of these patients were considered fit for return to work, and 55 % had chest radiographs showing complete resolution.

Of patients deemed ill enough to be admitted to the intensive care unit, the mortality rate is significant. Although the British Thoracic Society study of CAP managed on the Intensive Care Unit included a third of patients that were previously completely fit and a similar number under 44 years of age, the overall mortality rate was 48 %. The majority of the deaths were due not to respiratory failure but to multi-system organ failure, raising the possibility that deaths from CAP are due not so much to the bacterial cause of the pneumonia, but to an inappropriately aggressive host inflammatory response causing

'bystander' damage not only to the lungs themselves, but also to other organs, particularly the kidneys and the cardiovascular system.

In support of this, in bacteraemic disease, penicillin is able to markedly reduce mortality after day 5 of disease compared to no treatment or treatment with immune serum, but it is unable to prevent earlier deaths (Austrian and Gold, 1964). Penicillin is extremely active against Strep pneumoniae, and it is likely that all organisms would be killed in a matter of hours rather than days. Although toxic products produced by the bacteria could continue to cause injury, it is possible that the failure of penicillin to prevent early death is due to an uncontrolled inflammatory cascade triggered in the patient (Johnston, 1991).

Among the survivors of patients requiring intensive care treatment, over 50 % make a full recovery, with evidence of residual airways damage in 10 %, and pulmonary parenchymal damage (the microbiological aetiology for which was not reported) in just 6.5 % (The British Thoracic Society Research Committee and the Public Health Laboratory Service, 1992).

1.5 Nosocomial pneumonia

1.5.1 Incidence

The diagnosis of nosocomial pneumonia (NP) may not be easy, requiring invasive measures that are far from perfectly sensitive or specific for the diagnosis of pneumonia (Bamberger, 1988, Griffin and Meduri, 1994, Torres, 1991).

Nevertheless, it is clear that NP is common. It occurs in 0.6 % to 1.1 % of hospital patients (Haley et al, 1981 and Hughes et al, 1983), or about 300 000 patients per year in the USA (Gross, 1987). In intensive care units it is even more common. Four prospective studies have shown rates of NP in patients mechanically ventilated for more than 48 hours of 23 %, 21.9 %, 21 % and 24 % respectively (Jimenez et al, 1989, and Rello et al, 1991, Craven et al, 1986, Torres et al, 1990). George found that the incidence of nosocomial

pneumonia in patients on a medical intensive care unit was 0.9 cases per 1000 patient-days in unventilated patients, but 20.6 cases per 1000 patient days in mechanically ventilated patients (George, 1995).

Other groups may also be at particularly high risk, either because of their primary disease or because of the treatment they must receive. For instance, there is an incidence of NP of around 20 % after upper gastrointestinal surgery (Vickers, 1982), neurosurgery (Dauch et al, 1989) and bone marrow transplants (Pannuti et al, 1991), whilst 4 % of patients with solid organ transplants develop NP (Mermel and Mai, 1990).

Patients at risk of aspiration will also show a higher incidence, even when the aspiration is not clinically obvious (Kingston et al, 1991).

1.5.2 Bacterial Aetiology

NP has a much more variable bacteriological cause than CAP, including the presence of polymicrobial infection. The patient group, the type of hospital and the country of the study will have significant effects on the exact pathogens encountered. Individual studies have shown that gram negative rods are the most frequent pathogens (e.g. Hughes et al, 1983, Bartlett et al, 1986, Bryan and Reynolds, 1978), and this is confirmed by the overall figures for the USA showing the following frequencies of isolation (Horan et al, 1986):

Pseudomonas spp.	16.9 %
Staphylococcus aureus	12.9 %
Klebsiella spp.	11.6 %
Enterobacter spp.	9.4 %
Escherichia coli	6.4 %
Serratia spp.	5.8 %
Proteus spp.	4.2 %

The remainder of this thesis emphasises E. coli as a typical member of the gram negative rods so frequently involved in NP.

1.5.3 Outcome

In hospital-wide surveys of NP, crude mortality rates have been very high (25 %, 30 % and 36.6 %, Louie et al, 1991, Leu et al, 1989, Celis et al, 1988, respectively). In the study of Leu et al, 1989 case-control matching suggested an attributable mortality of 33 %. though this failed to reach statistical significance. An increase of 7 days in hospital stay was clearly necessary in survivors.

Still higher crude mortality rates have been encountered on intensive care units (55 %, 33 %, 42%, and 54.2 %, Craven et al, 1986, Torres et al, 1990, Rello et al, 1991, Fagon et al, 1993, respectively). Rello et al, 1991, found that the mortality rate of intensive care patients with or without NP was very similar at 42 % and 37 % respectively, but Torres et al, 1990 found mortality rates of 33 % versus 19 %. Fagon et al, 1993, compared the outcome in patients in Intensive Therapy Units with NP with that of a carefully matched control group, and showed an statistically significant attributable mortality of 27.1 %. This is of a very similar magnitude to the study of Leu et al, 1989, for NP throughout a general hospital population.

In summary, NP is common, occurring in about 1 in 1000 hospital patients. Best estimates suggest that about 1 in 3 of these will die as a result of their pneumonia.

1.6 The lung's defences

Each human lung has a surface area of 70 m^2 (Murray, 1986) which is in direct continuity via the bronchi, trachea, pharynx and mouth with the external environment. In excess of 10,000 litres of air will be respired per day and this may contain thousands of bacteria per cubic metre (Samet et al, 1987). A variety of defence mechanisms have evolved to cope with this.

In addition to potential airborne pathogens, the lung also has to cope with aspirated material from the oropharynx. Even in normals, 40 % aspirate radiolabel from the

oropharynx during sleep (Huxley et al, 1978), and this figure rises to 85 % in the severely ill. It is generally felt that defences are usually adequate to challenge by respired pathogens except in particular cases (Legionella and viral infection), but that pneumonia usually results when the defence to aspirated material is overcome. This may be either because of a large infective load or because of poor host defences (Coalson, 1995, Levine and Niederman, 1991, Nelson et al, 1995). Three orders of magnitude more bacteria are required to cause pneumonia when administered experimentally by aerosol than by bolus inoculation into the trachea (Ansfield et al, 1977).

1.6.1 Nasopharynx and oropharynx

The convoluted path of air flow through the nasopharynx ensures that all but the smallest particles will be trapped in this way before entering the lungs (Newhouse et al, 1976).

The normal oropharynx is colonised with 10^7 to 10^{10} bacteria per ml. The ability of other bacteria to survive here is limited by salivary flow, desquamation of the epithelium and local secretion of IgA, IgG and complement (Mackowiak, 1982). Normally, gram negative bacteria are not present (Johanson et al, 1969). Even normal subjects gargling with suspensions of 10^8 per ml Klebsiella pneumoniae, Proteus mirabilis or E. coli clear these bacteria within 6 hours (LaForce et al, 1976).

1.6.2 Trachea and bronchi

The normal lower respiratory tract is sterile (Lees and McNaught, 1959). Bacteria are mechanically cleared from the respiratory tract by the mucociliary escalator (see Levine and Niederman, 1991). Defects in the mucocilliary escalator leading to an increased risk of infection (Cammer, 1980).

1.6.3 Terminal airways and alveoli

A variety of soluble products within the alveolar lining fluids have antibacteriological properties. These include IgA, IgG, lactoferrin, free fatty acids and lysozyme (Coonrod, 1986). However, the greatest antimicrobial defence to low levels of bacteria in the normal lungs is provided by alveolar macrophages. (Fels and Cohn, 1986, Goldstein et al, 1974, Green and Kass, 1964, Hocking and Golde, 1979, Rylander, 1968). The alveolar macrophages are able not only to ingest bacteria and kill them intracellularly, but also to produce reactive oxygen species and other toxic metabolites that kill bacteria extracellularly (Brieland et al, 1982, Fantone, 1987, Jonhnston 1988, and Nathan, 1987).

It has long been recognised that, although resident cells of the alveolus may successfully defend the lung against relatively minor challenge, larger challenges are met by the recruitment of further phagocytic cells in the form of neutrophils and monocytes from the bloodstream (Briscoe, 1907, and Onofrio et al, 1983). Aspects of this neutrophil recruitment and their subsequent removal are the subject of this thesis.

1.7 Pathogenesis and pathology of community acquired pneumococcal pneumonia, with special reference to pneumococcal pneumonia

1.7.1 Epithelial attachment

Strep. pneumoniae is spread from person to person by aerosol. 40 % of normal subjects harbour the bacterium in their nasopharynx without any deleterious effect (Austrian, 1986). In some, it is able to advance further into the respiratory tract to cause disease. This was appreciated long ago by Metchnikoff (Metchnikoff, 1905);

"People who carry the Pneumococcus on their mucous membranes may remain for long without being attacked by fibrinous pneumonia or any other maladies due to this micro-organism. But often, in consequence of some special circumstance, a cold for example, the refractory state gives way to a more or less marked susceptibility"

Why this occurs in some cases but not in others remains unclear, but as previously noted this will depend on the balance between bacterial load and efficacy of host defence when small amounts are aspirated. It has also been noted that invasion tends to occur soon after the colonisation by a new serotype (Gray et al, 1980, Hodges and MacLeod, 1946). Of importance is the ability of Strep. pneumoniae to adhere to and enter cells of the respiratory tract. Some, but not all, Strep. pneumoniae are able to penetrate A549 cells, a human alveolar carcinoma line (Talbot et al, 1996). Another group could not show that the bacterium was able to invade resting A549 cells, but demonstrated increased adhesion with invasion into A549 cells after cytokine pre-treatment of the cell. In this case the Strep. pneumoniae adhere to the platelet activating factor (PAF) receptors. Avirulent strains were unable to adhere in this way (Cundell et al, 1995), suggesting that it is of critical importance to the establishment of disease.

1.7.2 Virulence factors

Strep. pneumoniae produces a number of factors that may be of importance in virulence. The capsular polysaccharide is clearly important, different serotypes having different propensity to cause disease. Pneumolysin has the capacity to injure endothelial cells in vitro (Rubins et al, 1992). It may activate complement (Paton et al, 1984), and at low doses causes neutrophil chemotaxis and granule release, whilst at high doses it lyses neutrophils and platelets (Johnson et al, 1981). Pneumolysin sufficient pneumococci survive better within mouse lung and are more rapidly invasive, producing bacteraemia more rapidly. Addition of pneumolysin to inoculates of pneumolysin deficient strains enhances survival and invasion. Both complement activating and cytotoxic activities seem to be important in this effect (Rubins et al, 1995). Pneumococci also produce hydrogen peroxide, which was shown to be damaging to alveolar epithelial cells *in vitro* and is well known to be highly toxic *in vivo* (Duane et al, 1993). As they are catalase negative, this also means that

Strep. pneumoniae are easily killed when phagocytosed, even in patients with chronic granulomatous disease whose granulocytes are unable to generate reactive oxygen species themselves (Johnston and Newman, 1977). Strep. pneumoniae strains with insertional inactivation of either pneumolysin or autolysin had greatly reduced virulence when assessed by survival of mice after intraperitoneal injection of the deficient organisms (Berry et al, 1992), and it seems that both factors (which are released during lysis of bacteria) are important in establishing pneumonia. The manner in which these different elements interact to cause disease is not well defined.

1.7.3 Pathology

Typical non-fatal pneumococcal pneumonia is an archetypal resolving inflammation. Initially, there is congestion and hyperaemia of the lung, with exudation into the alveolar spaces, followed by microhaemorrhages of red blood cells, producing what is classically described as 'red hepatisation'. Next, there is an influx of phagocytic cells to produce grey hepatisation. Initially these are neutrophils, followed by mononuclear cells. Later, resolution follows (Loosli, 1940, Wood, 1952). Not all areas of the pneumonic lung will be at the same pathological state simultaneously as the disease spreads, but rather each area passes through these stages.

Classical series of untreated pneumococcal pneumonia showed 'delayed resolution' (failure of resolution of clinical symptoms within the expected two weeks) in only about 4 % (McCrae, 1910). Three post mortem studies showed evidence of fibrosis in 0.6 %, 7.6 % and 0.7 % respectively (Chatard, 1910, Lord, 1925, and Fränkel, personal communication to Lord, 1925).

The normal lung tissue is seen to be composed of a very fine network of delicate tissue. During the height of pneumonia it is solidly packed with neutrophils, which have great potential to cause damage (Haslett et al, 1989a, Henson and Johnson, 1987, Stockley,

1995, Weiss, 1989). It is quite remarkable that following pneumococcal pneumonia, the apparently delicate lung is usually restored completely to normal (Johnston, 1991).

1.8 Pathogenesis and pathology of nosocomial gram negative pneumonia, with special reference to E. coli pneumonia

1.8.1 Epithelial attachment

The ability of bacteria to bind to respiratory epithelium increases in disease. This has been shown to directly correlate with colonisation of the upper respiratory tract with gram negative bacteria (Johanson et al, 1979). Furthermore, in a group followed before and after major surgery, those showing a less than twofold increase in *in vitro* adherence had no respiratory tract colonistion with gram negative bacteria. By contrast, in the group with a greater than twofold increase in adhesion *in vitro* , 69 % became colonised. (Johanson et al, 1980). Colonised patients have an eightfold increased risk of pneumonia (Johanson et al 1972).

Decreased stomach acidity associated with the use of antacids and H_2 blockers may allow the stomach to become an important reservoir in NP. In 60 mechanically ventilated patients, no case of pneumonia was seen in the 8 patients whose gastric and oropharyngeal growths were different, but more than 50 % of the remainder developed gram negative pneumonia. The number of bacteria cultured from the stomach correlated with gastric pH (Moulin et al, 1982). This has led to studies of the use of sucralfate, a gastric mucosal protective agent with no effect on pH. A meta-analysis showed the rate of NP to be lower in patients treated with sucralfate than H_2 blockers (Cook et al, 1991, and Tryba, 1991) although whether this is due directly to an effect on pH, or to a direct antimicrobial action of sucralfate is disputed.

Attempts have been made to prevent NP by selective decontamination of the gastrointestinal tract, using various regimes. Meta-analysis of a number of studies showed clear efficacy in reducing the risk of NP (Vandenbroucke-Grauls and Vandenbroucke, 1991), but disappointingly, no benefit was demonstrated on overall mortality. This is perhaps not surprising, given the difficulty in clearly demonstrating significant attributable mortality in NP, as discussed above. It seems likely that benefit will be confined to a subgroup with potentially remediable underlying disease (Van Saene et al, 1992), demanding a large scale trial to demonstrate. Nevertheless, these trials provide strong evidence for the important rôle of gram negative bacteria from the gastrointestinal tract in the pathogenesis of NP.

Even if bacteria resident within the patient's gastrointestinal tract are of prime importance, aspiration may not be the only route of pulmonary infection. A prospective study of NP in intensive care unit patients identified stress ulceration, illness severity and stomach intramucosal pH as most predictive of NP. Although use of H_2 blockers analysed independently were predictive of NP in a multiple regression analysis, inclusion of this risk added nothing to the predictive value of the previously enumerated risks. The three major risks are all associated with loss of gastrointestinal mucosal integrity. The authors speculated that this could indicate that bacterial translocation from the gut lumen and haematogenous spread is important (Fiddian-Green and Baker, 1991). This mechanism may contribute, but it seems more likely that demonstrated frequency and risk of aspiration make it the most important factor.

Thus it seems that in ill hospitalised patients, particularly those mechanically ventilated, there is overgrowth of gastrointestinal gram negative rods within the stomach. Epithelial changes in the pharynx allow these to colonise upwards, following which they may be aspirated to cause NP.

1.8.2 Virulence factors

E. coli virulence factors in extrapulmonary disease have been relatively well studied. In the urinary tract, pilus associated with pyelonephritis, haemolysin, aerobactin and cytotoxic necrotising factor are among those shown to be important (Yasamato et al, 1995). In diarrhoeal diseases, E. coli may produce a variety of exotoxins and specific adhesion factors are of importance (Blanco et al, 1991 and Cassels and Wolf, 1995).

There have been far fewer studies examining virulence factors of E. coli within the respiratory tract. Respiratory infections were included in an examination of clinical isolates. E. coli isolated from the respiratory tract showed the presence of haemolysin and adhesins in 84 % of cases, infecting a large number of patients with apparently intact host defences. Many hosts of infection with E. coli deficient in adhesins and haemolysin had impairment of their defences, illustrating the interplay between bacterial virulence and disease (Maslow et al, 1993).

E. coli haemolysin causes increased pulmonary artery pressure and increased filtration by a mechanism that can be primed with endotoxin and involves the co-operation of neutrophils in generating thromboxane A2 and leukotrienes (Grimminger et al, 1990, Seeger et al, 1991, Walmarth et al, 1994).

In addition, E. coli contain large amounts of endotoxin, which may have a damaging or activating effect on a wide range of cells.

1.8.3 Pathology

In general, the pathological appearances are the same in gram negative nosocomial pneumonia as in pneumococcal pneumonia. The exception to this is that Pseudomonas aeruginosa may produce a characteristic lesion called "Pseudomonas vasculitis" in which large numbers of bacteria cluster around and invade small vessels (Nash, 1989). In addition, gram negative pneumonia may cause necrosis of the alveolar walls to give multiple small

abscesses (Colason, 1995). This represents much more serious damage than is usually witnessed in pneumococcal pneumonia where the alveolar walls characteristically remain intact (Johnston, 1991). This initial damage is of great importance as it is likely to lead to increased residual damage and scarring.

1.9 Bacterial clearance and the pulmonary cellular response

1.9.1 Bacterial clearance

Bacteria deposited in the lung by aerosol are usually cleared quite rapidly. Using a combination of radiolabelling of bacteria and bacteriostatic inhibition of multiplication, the viable bacteria remaining in the lungs of mice were found to be determined by the sum of bulk removal, *in vivo* replication and killing (Jay et al, 1976a). Physical removal accounted for 7 -12 % of bacteria over 4 hours in all bacterial species studied. Killing of Strep. pneumoniae amounted to 87% per hour, whilst that of E. coli was 18 % per hour. The doubling time of Strep. pneumoniae and E. coli were 310 and 212 minutes respectively. The net result was that after 4 hours, 7.3 % of deposited Strep. pneumoniae were viable whilst 88.5 % of E. coli were viable. Clearly, the pattern and mechanisms of clearance depended on the species of bacteria.

Bacterial clearance rates also depend on the magnitude of the initial inoculate. More Staphylococcus aureus were cleared from the lung after 4 hours following a low dose rather than a high dose exposure. The opposite was true for Klebsiella pneumoniae and, whilst low doses of Pseudomomas gave net clearance, high doses led to net proliferation (Toews et al, 1979).

Clearance of aerosol-deposited Strep. pneumoniae and Pseudomonas aeruginosa were greatly impaired in hypocomplementaemic mice, whereas clearance of Klebsiella pneumoniae and Staphylococcus aureus were unaffected (Gross et al, 1978).

In addition, clearance of viable pneumococci varies depending on host species (Coonrod et al, 1990). Clearance was faster in rabbits than guinea pigs or rats. In the latter two species, extracellular killing was important, whilst in the former intracellular killing was predominant *in vivo*. Both mechanism and kinetics of bacterial killing by alveolar macrophages have also been shown to depend on the species source of the macrophages *in vitro* (Nguyen et al, 1982).

Clearly bacterial clearance is not a stereotyped process, but relies on a dynamic interaction between the specific bacteria and host. This is further illustrated by the cellular response of the lung to bacterial challenge.

1.9.2 Cellular response to bacterial challenge to the lung

After aerosol exposure to Staphylococcus aureus, mice failed to show an increase in intrapulmonary neutrophils. Klebsiella pneumoniae and E. Coli produced a marked neutrophil influx as early as 2 hours after exposure (Pierce et al, 1977). Over a longer time course, exposure to aerosolised Staphylococcus aureus was found to produce a modest increase in alveolar macrophage number, without any change in the number of neutrophils. By contrast, Pseudomonas aeruginosa produce a mild initial rise in alveolar macrophages, followed by a marked increase in neutrophils. Finally, over several days there was an increase predominately in peroxidase positive mononuclear cells, representing recently recruited monocytes (Sordelli et al, 1985).

The relative lack of importance of neutrophils in clearance of Staphylococcus aureus was demonstrated by the finding of unchanged clearance rates in mice rendered neutropaenic by anti-granulocyte globulin. By contrast, neutropaenia impaired the clearance of Klebsiella pneumoniae and Pseudomonas aeruginosa (Rehm et al, 1980).

The magnitude of innoculum was also shown to be important, with the number of neutrophils recruited being directly related to Streptococcus pneumoniae challenge (Toews et al, 1979).

The pattern of host response is not necessarily directly related to bacterial persistence. Four different patterns of clearance were found among 13 different strains of Pseudomonas aeruginosa, and these produced a variety of patterns of lung histology. However, no correlation between the clearance rate and histological appearances could be discerned (Southern et al, 1970).

Therefore it is clearly true that the host response is not stereotyped, but varies with the size and type of challenge. The above studies were performed using aerosolised bacteria to deposit relatively modest numbers of bacteria. When challenged with boluses delivered into the more distal airways, which may be more directly relevant to pneumonia as discussed above, responses may differ. Thus, just 10^4 Streptococcus pneumoniae placed intrabronchially in mice were able to proliferate to 10^7 viable organisms in 48 hours (Ansfield et al, 1977). This contrasts markedly with the rapid clearance seen in aerosol exposure studies.

Clearance studies of Staphylococci also differ with the two techniques. As discussed above, aerosol exposure to Staphylococcus aureus results in a mild increase in alveolar macrophages only. With direct inoculation, 10^5 organism were promptly cleared with a selective increase in alveolar macrophages. 10^6 organisms cleared more slowly and there was a small neutrophil influx. 10^7 organisms were cleared very slowly and elicited a very marked neutrophil influx. 10^8 organisms provoked an intense neutrophil influx, despite which the organism proliferated and death often followed.

1.9.3 Summary

These studies show that bacterial clearance varies greatly with number and species of bacteria, type of administration and the nature of the host. The cellular response varies with all these factors. Therefore, there is no stereotypical interaction between the lungs and bacteria, but a very specific one depending on exact circumstances.

1.10 The neutrophil in host defence

1.10.1 Importance of the neutrophil in host defence

Although the response to bacterial challenge is variable at low doses, it is clear that at higher doses the recruitment of neutrophils is intimately associated with host defence. Indeed, their arrival is the hallmark of the early stages of pneumonia.

The importance of neutrophils to host defence is indicated by the high risk of infection in neutropaenic patients (Dale et al, 1979), and neutropaenia has been confirmed to adversely affect defence to pneumonia experimentally (Wood and Irons, 1946, and Skinsnes, 1948). In leukaemic patients, the risk of infection correlates with the degree and duration of neutropaenia (Bodley et al, 1966). In patients with chronic infection, infusion of G-CSF elevates the peripheral blood neutrophil count and this was associated with resolution of infection (Bonilla et al, 1989).

Functional disorders of neutrophil also give rise to increased risk of bacterial infection e.g. chronic granulomatous disease (Churnutte et al, 1974) and leukocyte adhesion deficiency (Anderson et al, 1985).

Normally, the mature neutrophil is an intravascular cell. If an infection occurs, neutrophils collect at the site of the infection and invade the tissues to provide defence, flooding in great numbers. This astonishingly rapid and specific process led Andrew to remark;

"In presence of local tissue attack.... we find that the polymorphonucelar phagocytes converge upon this spot from all over the body, and this with truly remarkable rapidity. So prompt is the action, so purposeful its aim, that it is at first difficult to believe it other than an intelligent response on the part of the active agents"
(Andrew, 1910).

Although he continues by noting that members of the local constabulary converge promptly at sites of disorder, and thus that directed behaviour is not necessarily a sign of intelligence, he identifies well the high degree of specificity of control of neutrophil kinetics that this implies!

In order for these processes to occur, the normal circulating neutrophil must be detained at the site of infection, adhere to the vascular endothelium there, and migrate through. To accomplish this, substances activate the neutrophil to change its stiffness and its adhesion properties, whilst simultaneously endothelial adhesivity is specifically modulated. Chemicals are elaborated which direct neutrophil migration, either as soluble factors (chemotaxins) or as fixed factors (haptotaxins).

1.10.2 Neutrophil damage to the host; the paradox of inflammation

Although neutrophils are very clearly required for host defence, they contain a large armamentarium including oxidants and proteases (Root and Cohen, 1981). These have the potential not only to damage invading bacteria, but also (as they may be liberated extracellularly during inflammation, Schraufstätter et al, 1984) to cause 'bystander' damage to host tissues (Fujishima and Aikawa, 1995, Haslett et al, 1989a, and Henson and Johnston, 1987).

An example of this was provided in a model of Pseudomonas aeruginosa infection produced by intratracheal instillation in guinea pigs. Low (10^4) or high (10^8) bacterial inoculates were used in normal animals, animals rendered neutropaenic by cyclophosphamide and in animals rendered neutrophilic by G-CSF (Terashima et al, 1995). With low bacterial inoculates, persistence of viable bacteria was greater in neutropaenic

animals in which pulmonary epithelial permeability was also greatest. At high bacterial inoculates, bacterial permeability was highest in neutrophilic animals. There was a high mortality only in this group.

This suggests that, at low inoculates, the predominant lung damage was consequent to direct effects of the bacteria. A moderate neutrophil influx was required to clear the organisms and protect against this. However, at high dose in the presence of pre-existing neutrophilia such a profound influx of neutrophils (which may also have been partially activated by G-CSF) was produced that the predominant lung damage was produced by the neutrophils themselves. Once again, the dynamic relationship between the invading organism and the inflammatory response is well illustrated.

The neutrophil has been suggested to play a rôle in tissue damage in a wide range of diseases in which its recruitment may be regarded as inappropriate or excessive, both within the lung (in diseases such as asthma, chronic bronchitis, pulmonary fibrosis and ARDS, Reynold, 1987, Stockley, 1995) and elsewhere (such as in rheumatoid arthritis, ischaemia/reperfusion injury, various skin diseases, and ulcerative colitis, Weiss, 1989).

Neutrophils may even be important in host damage at a site distant from the original insult, such as in ARDS (Donnelly and Haslett, 1990, Repine and Beechler, 1991), in acid aspiration (Goldman et al, 1993) and in ischaemia-reperfusion (Simpson et al, 1993).

Neutrophil tissue burden must be of sufficient magnitude to protect against invading bacteria whilst at the same time not being so excessive as to cause host injury. A dramatic example of this is provided by the 'vicious circle' hypothesis of bronchiectasis (Cole and Wilson, 1989). Initial bronchial infection gives rise to bronchial damage secondary to inflammation, causing disruption of the mucocilliary escalator. This predisposes to further infection, another cycle of inflammation and further damage. Repeated cycles lead to bronchial wall damage and scarring.

The requirement for fine control of neutrophil burden during pulmonary inflammation may be exercised at the level of neutrophil production, intravascular trapping in the lungs, adhesion to the vascular endothelium, directed migration through the vessel wall into the interstitium and airspaces, and clearance from extravascular areas.

1.11 Normal neutrophil kinetics

1.11.1 Development

Neutrophil granulocytes are derived in the bone marrow from pluripotent stem cells. This process takes around 6 days in normal physiological conditions (Price et al, 1992). During development, they develop a large antibacterial armamentarium, contained in granules within the cytoplasm. Primary or azurophilic granules' contents include myeloperoxidase, lysozyme, and neutral serine proteases such as elastase, cathepsin G and proteinase 3, acid hydrolases, defensins and bacterial permeability increasing factors (Bainton and Farquhar, 1966, Baggiolini and DeWald, 1984). Secondary or specific granules contents include lysozyme, collagenase, lactoferrin, and supplies of receptors for FMLP, iC3b, and laminin. Granulocytopoiesis is enhanced by granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte stimulating factor (GM-CSF) and interleukin-3 (IL-3) (see Clark and Kamen, 1987, and Weisbart, 1989). G-CSF is a product of T lymphocytes, endothelial cells, fibroblasts and macrophages. GM-CSF is produced by monocytes and fibroblasts. IL-3 is a T cell product. These substances also have a modulatory effect on mature blood cells. Their receptors are also expressed on cells outside the haematological system (see Gasson, 1989), implying further actions.

The pre-eminent rôle of G-CSF in normal granulopoiesis is indicated by the binding of marked neutropaenia in G-CSF gene knock-out mice (Lieschke et al, 1994).On the other hand, GM-CSF gene knock-out mice show no significant perturbation of haemopoiesis.

Surprisingly, they do show characteristically abnormal pulmonary pathology (Stanley et al, 1994).

Furthermore, it is known that new-born mice clear intrapulmonary E. coli very poorly. In adults challenged thus, there is a granulocytopaenia at 4 hours, but the neutrophil count returns to or past normal by 24 hours. By contrast, although new-borns exhibit the initial granulocytopaenia, there is no recovery over 24 hours. The inability to accelerate neutrophil production over this period is correlated with failure to increase total G-CSF mRNA in liver, spleen or lungs (Liechty et al, 1993).

1.11.2 Circulating life

Once they have matured in the bone marrow, neutrophils are released into the circulation where they have a half-life of 4 - 7 hours (Cohen et al, 1981 and Doershuk et al, 1987). Damaged neutrophils are cleared more rapidly. At the end of their life span, neutrophils are cleared by cells of the mononuclear-phagocyte system. In physiological rather than pathological states, the number of extravascular neutrophils is very small. Morphometry shows that there are fewer than 10^6 intra-alveolar neutrophils in the rabbit (Cohen et al, 1979). There was no evidence of neutrophils leaving the lungs via the lymphatics, implying that neutrophils were disposed of locally.

The circulating half-life of the neutrophil is of the same order as the life span of the neutrophil *in vitro* before the onset of apoptosis. Apoptotic neutrophils can be recognised and phagocytosed by macrophages *in vitro*, and this may well represent a normal physiological process. There is some support for this from the examination of the kinetics and distribution of BrdU labelled cells in the rabbit (Bicknell et al, 1994).

1.11.3 Distribution of circulating neutrophils

The lungs are an important site of this temporary sequestration. Measurement of cells entering and leaving the lungs shows that there is rapid release of neutrophils after infusion

of adrenaline, with induction of peripheral neutrophilia, whilst LPS produces the opposite change (Ambrus and Ambrus, 1959, Athens et al, 1961), confirmed in humans using external scintigraphy (Muir et al, 1984)

Morphological studies have shown a tenfold concentration of leukocytes compared to erythrocytes in small pulmonary vessels, in *in vivo* and *ex vivo* dog preparations (Hogg et al, 1988 and Perlo et al, 1975). The latter study found that the leukocyte excess was predominantly due to mononuclear cells, possibly due to extensive extracorporeal manipulation. Retention of cells was inversely related to the perfusing pressure, suggesting that the lungs were acting as a cellular sieve.

Analogous findings have been found *in vivo*. Inflation of a balloon catheter in the inferior vena cava of dogs reduces the perfusion pressure to the lungs and increases intrapulmonary neutrophil retention (Thomassen et al, 1984), and a similar mechanism is also likely to explain the effect of adrenaline and exercise.

More direct measurement of this effect was performed using ^{99m}technetium labelled erythrocytes and ^{111}indium labelled neutrophils (Martin et al, 1987, and Doerschuk et al, 1987). Regional blood flow was directly related to pulmonary neutrophil retention, being greater at slower flows. The marginated neutrophil pool was at least twice that of the circulating pool and there was a turnover of cells within the marginated pool of around 1 % per second.

In a dog with a pulmonary window, videomicroscopy of FITC labelled neutrophils demonstrated that they were delayed in pulmonary capillaries. Neutrophils had a transit time of < 2 seconds to > 20 minutes, with a median of 26.1 seconds. Cells were observed to be held up at discrete points rather than passing evenly and steadily through the capillaries (Lien et al, 1987) confirming that the sequestered or marginating pool of neutrophils exists because the lung acts as a sieve, trapping individual neutrophils. This situation differs markedly from that observed in the systemic circulation, where the neutrophils appear to

pass more freely through the capillaries. However, neutrophils are displaced from the centre of post-capillary venules by axial flow of erythrocytes, and those held for a moment against the vessel walls are seen to roll for some moments, adhering temporarily to the endothelium, before passing on (Schmid-Schönbein et al, 1980).

The relative size of the neutrophil and pulmonary capillaries have been compared in humans, dogs and rabbits. 38 %, 67 % and 61 % of capillaries were found to be less than the mean spherical diameter of the neutrophil (Doerschuk et al, 1993). During its passage through the pulmonary capillaries, the neutrophil is likely to have to deform, and indeed, in this study, intra-capillary neutrophils were seen to adopt an ellipsoid shape. In addition, pulmonary blood pressure is lower than systemic blood pressure (Von Euler and Lijestrand, 1946) and, in the lungs, flow is pulsatile (Lee and DuBois, 1955). These factors together mean that the ability of the neutrophil to deform in order to pass through small capillary segments is likely to be critical in the lung. Studies of passage of erythrocytes and neutrophils through pores *in vitro* suggest that the erythrocyte's ability to deform is three orders of magnitude greater than that of the neutrophil (Chien, 1985), and thus neutrophils will be held up and concentrated in the lung relative to erythrocytes.

This mechanism was demonstrated in a model system using ^{99m}Technetium labelled erythrocytes and ^{111}Indium labelled neutrophils. Their passage through filter pores with sizes equivalent to pulmonary capillaries was examined. Selective neutrophil retention was flow dependent (Downey and Worthen, 1988).

Individual neutrophil stiffness was later measured with a "cell poker". It was confirmed that, after treatment with FMLP, cells became stiffer, an effect that could be antagonised by inhibition of f-actin assembly. Neutrophil stiffness correlated with first pass retention by the lung (Worthen et al, 1989). An antibody to the adhesion glycoprotein CD18 failed to have any effect on either passage through the filter or first pass retention in the lungs.

Using pulmonary vascular lavage to recover sequestered neutrophils, it has been demonstrated that sequestered cells are on average stiffer than those from the peripheral circulation, whilst also expressing more CD18 molecules on the cell surface (Brown et al, 1995b).

In summary, neutrophils are normally produced in the marrow under the influence of G-CSF. They are released into the circulation, where they have a short half-life. As a consequence of their stiffness and size, a proportion are temporarily sequestered in the pulmonary circulation. As they age, they are removed from the circulation by cells of the mononuclear-macrophage system, probably after undergoing apoptosis.

1.12 Pulmonary sequestration of neutrophils during inflammation

1.12.1 Induction of sequestration by inflammatory mediators

During inflammation there is an increased sequestration of neutrophils within the pulmonary vasculature, caused by a variety of substances.

Increased pulmonary sequestration of ^{111}indium-labelled neutrophils has also been demonstrated after intravenous PAF (Hultkvist-Bengtsson et al, 1991), inhaled PAF (Tam et al, 1992), intravenous GM-CSF (Hovgaard et al, 1992) and zymosan activated plasma (Doerschuk, 1992). Morphological techniques show sequestered neutrophils after intravenous C5f are located in pulmonary capillaries (Downey et al, 1993).

^{51}chromium labelled neutrophils exposed to sufficient PAF *in vitro* to cause priming for superoxide release and enhanced expression of the CD11b adhesion molecule did not exhibit any increased pulmonary sequestration when reinjected into rats (Fontes et al, 1995).The initial sequestration of neutrophils after infusion of zymosan activated plasma was unaffected by antibody-blockade of the CD18 adhesion molecule, although subsequent washout from the lungs was accelerated by anti-CD18 antibody (Doerschuk, 1992). Initial sequestration is thus independent of β_2 integrins.

1.12.2 Increased sequestration in inflammation; neutrophil or endothelial changes?

Theoretically, changes in the neutrophil or the pulmonary vascular endothelium could mediate increased neutrophil sequestration in the lung.

The exposure of ^{111}indium labelled neutrophils to endotoxin *ex vivo* increased their sequestration when reinjected into rabbits with normal lungs (Haslett et al, 1987), showing that endothelial changes are not necessary for increased sequestration.

After infusion of LPS, sequestered neutrophils from pulmonary vascular lavage were more activated than peripheral blood neutrophils and showed an increased ability to bind to nylon columns, though it is not possible to say whether the increased adhesivity caused their sequestration (Williams et al, 1993).

^{111}indium labelled neutrophils were retained in the lung in excess of ^{99m}technetium labelled erythrocytes when simultaneously injected into normal rats (Brown et al, 1995a). This was more marked when cells taken from normal rats were injected into animals with lungs inflamed by instillation of Corynebacterium parvum. However, inflammatory neutrophils obtained by broncho-alveolar lavage were sequestered to an even greater degree when injected into normal rats. This suggests that neutrophil changes have more potential to affect sequestration than changes in the endothelium.

During pneumococcal pneumonia in rabbits, ^{51}chromium labelled cells were sequestered at 2, 4 and 8 hours after the onset of pneumonia. In control, non-pneumonic areas of the lung there was also sequestration of neutrophils at 1 and 4 hours. (Doerschuk et al, 1994). It is likely that the generalised sequestration reflects neutrophil activation, as endothelial changes are likely to be localised. In addition, there was increased localised sequestration, which could be due to rapid neutrophil changes at the inflammatory site, or local endothelial changes.

Overall, the balance of evidence indicates that, during inflammation, increased pulmonary sequestration is largely due to an effect on the neutrophil, with endothelial changes having a lesser rôle.

1.12.3 Neutrophil rheology and pulmonary sequestration during inflammation

As discussed above, neutrophil rheology seems to play an important rôle in sequestration of neutrophils in the lungs in physiological conditions. The stiffness of neutrophils can be modulated by a number of factors. *In vitro,* smoke exposed neutrophils become stiffer as a result of actin polymerisation induced by oxidants (Drost et al, 1992). This is reflected *in vivo* when smoking reduces the washout of ^{111}indium labelled neutrophils from the lungs (MacNee et al, 1989).

Sequestered neutrophils after C5f infusion have rearranged f-actin compared to intracapillary neutrophils in animals receiving saline, and are more spherical (Motosugi et al, 1996). This is likely to result in neutrophils that are mechanically detained in pulmonary capillaries. FMLP produced actin rearrangement increased neutrophil stiffness *in vitro,* which paralleled their retention in filters and their retention in lungs when returned after *ex vivo* treatment (Worthen et al, 1989).

Sequestered cells obtained by pulmonary vascular lavage after inflammation induced by Corynebacterium parvum show increased stiffness compared to cells obtained from the peripheral vascular pool (Brown et al, 1995b).

Colchicine caused an initial pulmonary neutrophil sequestration, followed by recovery in rabbits (Mueller et al, 1994), indicating a rôle for microtubule reassembly during normal passage through the lung. Colchicine failed to inhibit the increased neutrophil sequestration after Strep. pneumoniae instillation intra-bronchially. However, it inhibited migration from the vascular to the alveolar space. It is not possible to say whether this was due to an effect on the neutrophil or on resident lung cells.

Clearly, modulation of neutrophil rheological properties is of critical importance in their increased sequestration in the lung during inflation.

1.12.4 Adhesion molecules and neutrophil sequestration during inflammation

Adhesive interactions between the neutrophil and the vascular endothelium are mediated by a number of molecules. The three selectins are important. These are molecules possessing lectin-like domains. L-selectin is found on the neutrophil, whilst E- and P-selectin are found on activated endothelial cells. They bind to carbohydrate moieties, particularly sialyl-Lewis x and sialyl-Lewis a.

The β_2 integrins are heterodimeric molecules with a common β subunit (CD18) found on the neutrophil. They bind to the immunoglobulin-superfamily members ICAM-1 and ICAM-2, and additional uncharacterised receptors, on the endothelial surface. These molecules are also distributed among a variety of other cell types.

1.12.4.1 Selectins and pulmonary neutrophil sequestration during inflammation

In combined E- and P-selectin gene knockout mice there was a peripheral neutrophilia in the absence of infection, together with a concomitant increase in pulmonary neutrophil sequestration (Mizgerd et al, 1996).

P-selectin knockout mice show an increase in peripheral blood neutrophil count after infusion of adrenaline, suggesting that P-selectin is not necessary for physiological sequestration (Johnson et al, 1995). Mulligan et al, 1992, noted that fewer neutrophils were closely apposed to the pulmonary capillary wall after anti-P-selectin antibodies, but whether this pool is strictly the same as that defined functionally is unclear.

Neutrophil sequestration in guinea pigs after TNFα infusion was moderately reduced by pre-treatment with antibodies against sialyl-Lewis X and Lewis X (ligands for the selectins), as assessed by lung myeloperoxidase content (Lo et al, 1994).

In rats, the generalised sequestration of ^{111}indium labelled neutrophils in a number of tissues after burns (Hansbrough et al, 1996) was inhibited by anti E- and P- selectin monoclonal antibody. This global sequestration is probably not the same as the more specific pulmonary sequestration of neutrophils in other circumstances.

The lack of a demonstrable rôle for selectins in pulmonary neutrophil sequestration in gene knockout mice argues strongly against their importance in this process. The apparently contradictory efficacy of anti-bodies against selectins or their ligands in reducing sequestration could be due to experimental differences, or unsuspected *in vivo* cross reactivities of the antibodies, although compensatory mechanisms could be induced in gene knock-out mice. Overall, the rôle of selectins in pulmonary neutrophil sequestration seems at most limited.

1.12.4.2 Integrins and pulmonary neutrophil sequestration during inflammation

Sequestered neutrophils recovered by pulmonary vascular lavage have increased CD18 expression (Brown et al, 1995b) and increased adhesivity (Williams et al, 1993), suggesting that CD18 may play a rôle in sequestration. This has been supported by the ability of anti-CD18 antibodies to inhibit sequestration in a variety of circumstances; zymosan injection, following LPS (Miotta et al, 1991), during cardiopulmoanry bypass (Dreyer et al, 1995), after intraperitoneal LPS (Moriasaki et al, 1991) and after gut ischaemia/reperfusion (Hill et al, 1991).

Nevertheless, *in vitro* PAF induced up-regulation of CD18 did not increase neutrophil sequestration when the cells were returned to the circulation (Fontes et al, 1995). On the other hand, GM-CSF causes clear neutrophil sequestration that cannot be inhibited by anti-CD18 antibody (Yong et al, 1992).

Others have found CD18 dependence to vary with site or time. Generalised pulmonary neutrophil sequestration was CD18 dependent except in the portion of lung

directly exposed to acid, where anti-CD18 antibodies fail to inhibit neutrophil sequestration (Goldman et al, 1995). Pulmonary sequestration of ^{111}indium labelled cells after intravenous E. coli was CD18 independent after 4 hours, but became CD18 dependent after 24 hours (McCandless et al, 1994).

Doerschuk (1992) found that initial neutrophil sequestration due to infusion of zymosan activated plasma was CD18 independent, but that subsequent washout of these cells was accelerated by anti-CD18 antibody. She proposed a two-stage mechanism, where initial sequestration was CD18 independent, but the position of the cells within the pulmonary vasculature was stabilised by CD18 dependent mechanisms.

Studies showing CD18 dependent sequestration were generally performed over relatively long periods (several hours). Both mechanisms will be included, with overall CD18 dependence of sequestration, and it is reasonable to regard this as generally true. Nevertheless, other studies have clearly shown that expression of increased CD18 does not necessarily lead to sequestration, and that increased sequestration may in some circumstances be entirely CD18 independent.

1.13 Adhesion molecules in migration of neutrophils from the intra-vascular space

"A beneficial inflammatory reaction that leads to containment of infection may cause early tissue damage and later lead to fibrosis and disturbed lung function. Since nature has also recognised the importance of containment and limitation of inflammation, the exit of cells from the circulation into the tissues is subtly regulated at multiple levels"
Furfaro and Berman, 1992

1.13.1 Contrasts between systemic and pulmonary circulation

In order to combat infective challenges, neutrophils must migrate from their usual intravascular position and pass out into the tissues. In tissues supplied by the systemic circulation, this migration occurs from the post-capillary venules (Grant, 1974). As the

vascular space widens, there is axial streaming of the erythrocytes, tending to displace neutrophils to the periphery. Here, they exhibit a characteristic rolling against the endothelium (Atherton and Born, 1971, and Atherton and Born, 1973). Rolling increases in the presence of inflammation. Intermittently, neutrophils are seen to stop, and it is following these halts that migration occurs.

The two mechanisms were dissociated using intravital microscopy of rabbit mesentery. Anti-L-selectin antibody inhibited rolling. Anti-CD18 antibody had failed to inhibit the number of neutrophils that rolled (though mean velocity of rolling neutrophils increased), but inhibited subsequent firm adhesion (Von Andrian et al, 1989).

Since the rôle of L-selectin on the neutrophil in mediating rolling was first elucidated, it has also been shown that rolling is supported by E- and P-selectin on the endothelium (Abassi et al, 1993, and Jones et al, 1992).

It is hypothesised that the rapid adhesion mediated by selectins is important in conditions of significant sheer stress witnessed in blood flowing in venules. Once the neutrophils have been slowed and temporarily "captured" by selectin mediated adhesion, further firmer integrin mediated adhesion can occur, followed by emigration from the vascular space. The firm integrin mediated adhesions are only able to be established at low shear stresses (Lawrence and Springer, 1991, and see Butcher, 1991, Smith, 1992a).

A further elegant demonstration of the dissociation of these types of adhesion is provided by the infusion of neutrophils from humans with leukocyte adhesion deficiencies I and II into *in vivo* IL-1β activated rabbit mesentery. In leukocyte adhesion deficiency I there is a congenital absence of the CD18 β2 integrin, but normal selectin expression. In the rabbit mesentery preparation, these cells are seen to roll normally. However, they do not show the normal firm attachments. By contrast, cells from patients with leukocyte adhesion deficiency II do not present sialyl Lewis X, a ligand for endothelial E- and P-selectins, due to defective fucosyl-transferase activity. These cells do not roll and fail to adhere in

conditions of flow. However, if flow is diminished by cross clamping the supplying artery, neutrophils are seen to successfully adhere in near static conditions.

It has since been shown that L-selectin crosslinking actually increases CD18 adhesivity (as revealed in latex bead binding), demonstrating that, although the two types of receptors mediate different functions, they are not independent (Simon et al, 1995). This interrelationship was also seen in an activated rabbit mesentery preparation (Von Andrian et al, 1992). Neutrophil rolling was inhibited by anti-L-selectin antibody. Activation of neutrophils before infusion gave decreased rolling that paralleled the loss of L-selectin seen on cell activation. However, after brief (1-3 minutes) activation, the loss of L-selectin was incomplete. Though rolling diminished, cells that did roll had a strong propensity to progress to firm adhesion.

The situation in the lungs differs importantly from this. Careful morphometric analysis of inflamed rabbit lung has shown the site of neutrophil emigration is the capillary rather than the post-capillary venule (Downey et al, 1993). In addition, studies of neutrophils in pulmonary vasculature demonstrate that they move with intermittent stop/starts rather than the rolling observed in systemic circulation (Lien et al, 1987). As discussed, their sequestration in the lungs, both in physiological and pathological conditions, is primarily dependent on neutrophil rheological characteristics, because of the narrow pulmonary capillary bed (Doerschuk et al, 1993), the unusually low pressure (Von Euler and Lijestrand, 1946) and pulsatile flow characteristics of the pulmonary circulation (Lee and DuBois, 1955). Adhesion molecules play a minor rôle in initial sequestration. The overall effect of these mechanical constraints means that, in the pulmonary circulation, neutrophils are brought into close contact with the epithelium at low shear stresses without prior intervention of specific adhesive interactions. This very different mechanical relationship between the neutrophil and the endothelium in the pulmonary circulation compared to the

systemic circulation raises the possibility that the use of adhesion molecules may be significantly different in the two cases.

Once tethered to pulmonary vascular endothelium, the neutrophils are able to migrate out of the vascular space, probably passing between endothelial cells, then through pre-formed holes in the basement membrane. It has been suggested that these are normally covered by fibroblasts, and that the neutrophils 'lift' these away from the hole as they migrate through (Walker et al, 1995).

1.13.2 Selectins and neutrophil migration from the systemic circulation

L-selectin

L-selectin is clearly important for successful neutrophil emigration from the systemic circulation. Anti-L-selectin antibody inhibits neutrophil influx in rabbit peritonitis (Fortenberry et al, 1994) and L-selectin gene knockout mice have reduced neutrophil influx into thioglycolate broth induced peritonitis (Tedder et al, 1995).

P- and E-selectin

Anti-P selectin antibody alone has been reported to inhibit neutrophil influx following ischaemia and reperfusion of the liver (Garcia-Craico et al, 1995), heart (Chen et al, 1994) and ear (Winn et al, 1993), but not after limb ischaemia (Seekamp et al, 1994).

In other situations, P-selectin blockade has only inhibited neutrophil influx when E-selectin is not available as an alternative; there appears to be some functional redundancy. In peritonitis, anti-E or anti-P selectin antibodies only produced moderate inhibition of neutrophil influx, but influx was abolished by the two together (Bosse et al, 1994). Similar effects were observed in pleurisy (Henriques et al, 1996).

Although P-selectin knock-out mice showed decreased neutrophil rolling in response to peritonitis, the eventual effect on neutrophil influx was not reported (Johnson et al, 1995). Anti-P-selectin antibody inhibited early but not late neutrophil influx in response to

peritonitis and delayed type skin hypersensitivity in wild type animals. Neutrophil influx was normal in these models in E-selectin knock-out mice, but in these animals, anti-P-selectin antibodies abolished late as well as early neutrophil influx (Labow et al, 1994).

The time-dependence of P-selectin blockade in wild-type mice is likely to result from the fact that increased E-selectin expression depends on protein synthesis and thus takes several hours (Bevilaqua et al, 1989), whereas P-selectin is stored preformed in Wiebal-Palade bodies which can be translocated to the cell surface in minutes (McEver et al, 1989). If there is P-selectin blockade, E-selectin is only available as an alternative later on in inflammation.

Selectin expression not only evolves with time during inflammation, but also varies anatomically too. Activated endothelial cells may express E-selectin, tissue factor and thrombomodulin, but in baboon sepsis, expression of these is discordant in different tissues (Seekamp et al, 1994).

In summary, P- and E-selectin are important for neutrophil migration from the systemic circulation, but there is some functional redundancy. Their expression varies with place and time.

1.13.3 Selectins and neutrophil migration from the pulmonary circulation

L-selectin

Anti-L-selectin treatment has been shown to inhibit pulmonary neutrophil influx in a wide variety of circumstances. Oligosaccharides produced to block selectin binding, anti-L-selectin antibodies, and L-selectin/antibody chimeras reduce neutrophil influx after IgG deposition (Mulligan et al, 1993c, Mulligan et al, 1994a and Mulligan et al, 1993b). Burns and hindlimb ischaemia and reperfusion both produce local and pulmonary neutrophil influx that can be inhibited by anti-L-selectin antibodies (Mulligan et al, 1994b and Seekamp et al, 1994).

The only report of failure of L-selectin blockade to inhibit neutrophil influx is in Streptococcal pneumonia, when fucoidin failed to affect neutrophil influx (Mizgerd et al, 1996).

E- and P-selectin

There is much more variable data relating to the rôles of E- and P-selectin in neutrophil emigration from the pulmonary vasculature.

E-selectin is not expressed in normal lung, but is found in areas of neutrophil influx during pulmonary fibrosis (Nakao et al, 1995). An antibody against both E- and P-selectins inhibits pulmonary neutrophil influx when given before or soon after intravenous Pseudomomas aeruginosa (Ridings et al, 1995b and Ridings et al 1995a).

After hindlimb ischaemia/reperfusion, pulmonary neutrophil influx is inhibited by anti-E-selectin antibody alone (unlike neutrophil influx to the post-ischaemic limb), whilst anti-P-selectin inhibits early neutrophil influx only (Seekamp et al, 1994).

E-selectin chimeras inhibit pulmonary neutrophil influx after IgG immune complex deposition, but P-selectin chimeras have no effect, whilst after cobra venom factor, the opposite is true (Mulligan et al, 1993b).

In the ICAM-1/P-selectin double knockout mouse, although neutrophil influx to pneumococcal peritonitis is abolished, there is no abnormality of influx to pneumococcal pneumonia (Bullard et al 1995). In the E- and P-selectin double knock-out mouse, additionally treated with fucoidin to inhibit L-selectin, neutrophil influx to pneumococcal pneumonia is still normal (Mizgerd et al, 1996).

In summary, E- and P-selectin usually (but not always) play important parts in neutrophil influx to the lung, but their exact rôles dependent on the stimulus.

1.13.4 Integrins and neutrophil migration from the systemic circulation

There is no doubt that the β_2 integrins have a substantial rôle in adhesion and migration of neutrophils. The integrins are heterodimers, and the β_2 chain (CD18) can combine with α_L (CD11a) to give LFA-1, α_M (CD11b) to give Mac-1 or α_x (CD11c) to give p150,95, although the latter appears to play a minor rôle in adhesion.

In vitro, migration of neutrophils across filters is greatly inhibited by anti-CD18 and anti-CD11b, but anti-CD11a causes very little inhibition. Anti-CD11a and anti-CD11b each inhibit neutrophil migration across cultured endothelial monolayers by about 50 %, and their action together is additive (reviewed in Smith, 1992a).

Activation of cells leads to upregulation of Mac-1 number and function, whereas LFA-1 numbers can only be slightly increased. LFA-1 binds to two members of the immunoglobulin supergene family, ICAM-1 and ICAM-2, on endothelial surfaces. Mac-1 binds not only ICAM-1, but also other undescribed endothelial ligands (reviewed in Zimmerman et al, 1992).

Clinically there is no doubting the importance of CD18. Patients with a deficiency of this molecule show a marked propensity to develop life-threatening infections. These characteristically fail to show neutrophil infiltration, although there is a peripheral leukocytosis (Anderson and Springer, 1987).

The ability of anti-integrin antibodies to inhibit neutrophil migration to inflammatory sites *in vivo* has been studied in a multitude of models. In a review published in 1992, 10 studies of anti-CD18, 2 of anti-CD11b and 2 studies of anti-ICAM-1 effects on neutrophil recruitment from the systemic circulation to sites of inflammation were documented, all showing clear inhibition (Harlan et al, 1992). In addition, they tabulate 17 studies of anti-CD18, 2 studies of anti-CD11b and 3 studies of anti-ICAM-1 antibodies that have protected against inflammatory tissue injury (including ischaemia/reperfusion damage) in sites supplied by the systemic circulation, presumably because of inhibition of neutrophil

recruitment, and consequent decrease 'bystander' damage caused to the host by neutrophils. Since, there have been at least an additional 20 studies that have shown anti-integrin manipulations to have reduced neutrophil influx from the systemic circulation.

There have been some exceptions to these general findings. Neutrophil influx is not always CD18 independent in glomerulonephritis (Tipping et al, 1994, and Wu et al, 1993). Anti-CD18 antibody was unable to prevent neutrophil influx after hepatic ischaemia/reperfusion (Langdale et al, 1993).

Particularly interesting is a study comparing neutrophil influx in peritonitis caused by either Listeria monocytogenes or Salmonella typhimurium in mice. In the latter case, neutrophil influx was almost completely inhibited by either anti-CD18 or anti-CD11b antibodies. These antibodies were without effect on neutrophil influx due to Listeria monocytogenes (Conlan and North, 1994). If listeriolysin-negative strains of Listeria were used, the neutrophil influx became CD18 dependent. It is not clear whether the listeriolysin was inducing a CD18-independent pathway of neutrophil migration, or whether it was inhibiting the more frequent CD18 dependent one, necessitating recruitment of reserve pathways. Yet again, the dynamic interplay between host and challenging organisms in the determination of the inflammatory response is indicated.

The neutrophil response to peritonitis has also been studied with Escherichia coli and Streptococcus pneumonia (Mileski et al, 1990). Anti-CD18 antibody inhibited the recruitment of neutrophils to the peritoneal cavity in both cases. If the peritoneum was pre-primed by protease peptone injection 72 hours prior to bacterial challenge, neutrophil influx to Strep. pneumoniae became CD18 independent, whilst E. coli induced influx remained CD18 dependent. Lavage of the peritoneum after priming but before administration of Strep. pneumoniae restored CD18 dependency. Transplantation of the lavaged macrophages to a virgin mouse peritoneum and subsequent challenge with Strep. pneumoniae showed that CD18 independence could be transferred with the macrophages. Thus it seems that Strep.

pneumoniae interacts with macrophages to induce a CD18-independent pathway of neutrophil passage into the peritoneum.

In E. coli and protease peptone induced peritonitis, neutrophil accumulation assessed by peritoneal lavage neutrophil counts was inhibited by anti-CD18 antibody (Winn and Harlan, 1993). By contrast, at 24 hours, there was very little, if any, inhibition of neutrophil numbers, implying that CD18 independence had developed during the course of the inflammation. Inhibition of macrophage accumulation by co-administration of anti-CD49d antibody blocked the increase in both mononuclear cells and neutrophils at 24 hours. Neutrophils do not possess CD49d, so this implies that the inhibition of 24 hour neutrophil numbers seen when both antibodies were administered is secondary to the inhibition of mononuclear infiltrate. As in the case of Strep. pneumoniae, induction of CD18-independent migration is postulated to rely on the presence and action of local macrophages.

This experiment urges some caution in the interpretation of other studies of CD18-dependency of neutrophil migration. Almost without exception other studies that have overwhelming supported the role of CD18 in neutrophil migration have measured neutrophil accumulation after 3-12 hours. It remains a possibility that normal neutrophil migration in other forms of inflammation becomes neutrophil independent as a result of normal evolution of the inflammatory process.

In summary, in the overwhelming majority of studies, neutrophil emigration from the systemic circulation has been shown to depend on the β_2 integrins. Some stimuli may cause CD18 independent neutrophil migration, and this may develop in the presence of high numbers of local macrophages. In some cases, neutrophil migration may become independent of CD18 during the course of the inflammatory response, but how widespread this phenomenon is remains uncertain.

1.13.5 Integrins and neutrophil migration from the pulmonary circulation

Fewer studies have been undertaken of the importance of β_2 integrins from the pulmonary than the systemic circulation. The 1992 review referred to earlier (Harlan et al, 1992) tabulates 3 studies of anti-CD18 antibodies, one of anti-CD11b antibodies and 1 of anti-ICAM-1 antibodies on neutrophil recruitment. The results of these have been rather more variable than studies of neutrophil migration from the systemic circulation.

Intra-tracheal LPS produces a neutrophil influx in rats that is approximately 30 % inhibited by anti-CD11a antibody and 60 % inhibited by anti-ICAM 1 antibody (Tang et al, 1995). The effects of anti-CD118 and ant-CD11b antibodies were not reported, so it is unclear if complete inhibition of β_2 integrins would have been able to inhibit neutrophil influx more effectively.

IgG immune complex deposition causes neutrophil influx (Mulligan et al, 1995). Intravenous antibodies to CD11a and ICAM-1 were able to block this, although anti-CD11b antibodies given intravenously were without effect. When these antibodies were given intratracheally, anti-ICAM-1 still markedly inhibited neutrophil influx. However, anti-CD11a was now without effect whereas anti-CD11b markedly inhibited. This suggests that CD11a is important intravascularly, but as the neutrophil migrates from the circulation CD11b becomes important.

In lung injury due to infusion of cobra venom factor, neutrophil influx was inhibited by anti-CD11a, anti-CD11b and anti-ICAM-1 by 30 %, 50 % and 60 % respectively (Mulligan et al, 1993a).

Ozone induced neutrophil accumulation to the lung is also inhibited by anti-CD18 molecules in dogs (Li et al, 1992).

Anti-ICAM-1 and anti-CD18 inhibit neutrophil influx to the lung after PMA instillation, though anti-CD11a was without effect (Barton et al, 1989).

An early suggestion that CD18 mediated adhesion was not always comparable in the pulmonary and systemic circulations was provided by the finding that anti-CD18 antibody protects against neutrophil-dependent tissue damage after hypotensive shock in most tissues, but not in the lung (Vedder et al, 1988). In keeping with this, some cases of leukocyte adhesion deficiency type I show histological evidence of successful recruitment of neutrophils to the lung during pneumonia, despite the congenital lack of CD18 (Hawkins et al, 1992).

In an acid aspiration model in the rabbit, instillation of hydrochloric acid produces a local influx of neutrophils into the lung that is CD18 independent (Goldman et al, 1993). Interestingly, there was a neutrophil sequestration in the rest of the lung that was inhibited by CD18 blockade.

Direct pulmonary ischaemia/reperfusion using different experimental protocols has been found to be CD18 independent (Thomas et al, 1995) or CD18 dependent (Horgan et al, 1990). Whether timing or degree of ischaemia is critical in the difference is uncertain, but it is interesting that the requirement for CD18 may vary in such apparently similar circumstances.

Anti-CD18 antibody inhibited neutrophil influx to LPS (though not completely) but was without effect in neutrophil recruitment to Strep. pneumoniae (Doerschuk, 1990). Animals were also simultaneously challenged with sponges containing Strep. pneumoniae or LPS placed in the anterior abdominal wall. Neutrophil influx to these sites was abolished by anti-CD18 antibody in both cases. PMA was similarly studied. Neutrophil influx to this stimulus was completely CD18 dependent in both the abdominal wall and the lungs.

Lungs have a content of resident macrophages, and this may contribute to induction of CD18 independent pathways. Experiments performed in peritonitis (Mileski et al, 1990) , have supported the view that macrophages may contribute to induction of CD18 independent neutrophil migration pathways.

Further studies were undertaken in the rabbit. Neutrophil accumulation after 4 hours was shown to be predominately CD18 dependent for PMA, LPS, and live E. coli. Strep. pneumoniae produced largely CD18 independent migration, as did the instillation of supernatant from Strep. pneumoniae exposed alveolar macrophages. Emigration in response to PMA could not be inhibited by cycloheximide administered intravenously prior to the stimuli. By contrast, cycloheximide did block recruitment due to LPS and Strep. pneumoniae, suggesting that in these cases new protein synthesis was required.

Quantitative immunogold examinations demonstrated that, although in E. Coli pneumonia there is a greater than fourfold increase in ICAM-1 expression, there is no such increase in pneumococcal pneumonia (Burns and Doerschuk, 1994a). In addition, migrated cells show characteristic changes of activation in E. coli pneumonia, with decreased l-selectin levels and increased expression of CD-18. This was not found in cells that were still intravascular. By contrast, both intra- and extra-vascular neutrophils showed these changes in pneumococcal pneumonia, illustrating very different use of the repertoire of adhesion molecules (Burns and Doerschuk, 1994).

Simultaneous pulmonary and intradermal challenge with C5a and IL-1α were used to compare the CD18 dependence at different sites (Hellewell et al, 1994). Neutrophil emigration in the skin in response to both stimuli was completely CD18 dependent. In the lung, IL-1α induced neutrophil influx was partially inhibited by anti-CD18 antibody, whilst influx due to C5a was CD18 independent.

Acute Pseudomonas pneumonia produced neutrophil influx that could be inhibited by anti-CD18 antibody (Kumasaka et al, 1996). A second instillate given after 7 days to a different portion of lung gave a similar neutrophil influx with the same CD18 dependency. If instead the second administration was made into the same lobe as the first, there was only 15-20 % of the initial neutrophil influx. Neutrophil influx in this recurrent pneumonia was shown to be CD18 independent.

In summary, neutrophil influx in the lung is variably dependent on β_2 integrins. This depends on the stimulus, and whether it is a primary or recurrent inflammation. The exact timing of the stimulus may play a rôle. Neutrophil influx that is CD18-independent may require the participation of macrophages and new protein synthesis.

1.14 Chemotaxis

1.14.1 The concept of chemotaxis

As described above, during the inflammatory response the circulating neutrophil shows a remarkable ability to linger at the site of inflammation and adhere to the vessel wall. It then migrates from this site through the tissue to the source of the inflammation. Clearly there must be some mechanism to attract or steer the neutrophil in the correct direction. Although it is mechanistically possible to imagine a series of adhesions which would direct random neutrophil movement, it has long been the predominant view that neutrophils respond to some sort of chemical gradient within the tissues.

This process whereby a cell shows directed migration in response to a chemical substance is known as chemotaxis. The concept was first developed in the nineteenth century (see Harris, 1954). A variant on this has been more recently developed in which a chemical gradient is not obtained by soluble molecules, which might well be dispersed by blood and lymph flow *in vivo*. Instead, it is envisioned that chemicals are fixed onto the surfaces of cells or matrix proteins to establish a gradient, and cells migrate along this, the process of haptotaxis (Smith, 1992b).

By 1954 when Harris had published his review, it had been established that bacterial products could cause directed movement of neutrophils *in vitro* and *in vivo*. However, no specific substances with this property had been isolated. In particular, Harris was sceptical as to whether the body itself produced any chemical substance in response to bacteria that was itself a mediator of chemotaxis.

Since then, a variety of methods of studying chemotaxis *in vitro* have been developed (Bignold, 1988). Most commonly used are a variation on Boyden chambers, in which differing concentrations of a test substance are placed on either side of a filter, and the ability of neutrophils to migrate through the filter in response to a concentration is assessed. More sophisticated methods using cellular monolayers grown on filters may also be used.

These *in vitro* methods have been used to define a number of chemotactic substances.

1.14.2 Chemotaxins

Chemotaxins may be derived from the host or more or less directly from invading bacteria. The best known of the latter is formyl-methionyl-leucyl-phenylalanine (FMLP) (Marasco et al, 1984). This acts via specific receptors on the neutrophil cell surface, which have 7 transmembrane spanning domains and are G-protein linked. This has been found to be characteristic of a number of chemotactic receptors (see Caterina and Devreotes, 1991). Bacteria also produce a variety of less clearly defined chemotactic factors (Ras et al, 1990).

Leukotriene B4 is an arachidonic acid metabolite that has been found to produce potent chemotactic effects *in vitro* (Palmblad et al, 1981). This effect was maximal at 10^{-6} M. It also enhances adhesion to nylon fibres. This enhanced adhesion is an important aspect of chemotaxis. It appears to be mediated by fresh expression and upregulation of CD11b/CD18, and this expression seems to be carefully topographically controlled (see Smith, 1992b).

A second well studied lipid mediator of chemotaxis is platelet activating factor (PAF) (Goetzl et al, 1980). This chemotactic and neutrophil activating molecule is particularly interesting as it is found not only as a free molecule, but also on the surface of activated endothelial cells. Binding of PAF to the neutrophil causes shedding of L-selectin and upregulation of CD18 integrins. Thus when neutrophils are brought into close contact with endothelial cells (for example during selectin adhesion in the systemic circulation or due to

mechanical factors with or without selectin adhesion in the pulmonary circulation) they will bind PAF and develop the adhesive mechanisms necessary for firm adhesion (see Butcher, 1991, and Zimmerman et al, 1992).

The complement fragments C5a and $C5a_{des\ arg}$ are important chemotaxins generated by activation of complement (Fernandez et al, 1978). Clearly bacterial invasion is likely to be associated with generation of these products. Studies of hypocomplementaemic mice urge some caution, though. The clearance rate of Staphylococcus aureus was independent of the complement status of the animal. The clearance rate of Streptococcus pneumoniae and Pseudomonas aeruginosa were decreased (Hendrick et al, 1982). However, only in the former could this be shown to be associated with decreased neutrophil influx to implicate complement's chemotactic (as opposed to opsonic) actions.

A further cautionary note in extrapolation from these *in vitro* results is provided by study of the passage of neutrophils across naked filters, or filters with monolayers of either human umbilical cord endothelial cells (HUVEC), or kidney or lung epithelial cell lines (Casale et al, 1992). Although maximal chemotaxis to FMLP, PAF and LTB_4 was similar across each barrier, the rank order of potency depended on the nature of the barrier.

Tumour necrosis factor alpha is another host-derived factor demonstrated to posses chemotactic actions (Ming et al, 1987).

The overall relevance of these *in vitro* data is the finding that in 41 of 94 patients with recurrent infections, a defect of chemotaxis could be demonstrated (Ottonello et al, 1995).

Of the factors described so far, although all are chemotactic for neutrophils, they are also significantly chemotactic for other inflammatory cells. This is a problem that was identified quite a while ago by Harris, who, in summing up his 1954 review of chemotaxis, identified the following as the second of his three key questions about chemotaxins:

"How is the predominance of monocytes in certain lesions to be explained, if polymorphonuclear leukocytes and monocytes react chemotactically to the same stimuli?" (Harris, 1954)

The discovery in the 1980s of a new class of compounds, the chemokines, caused great interest as a consequence, as the chemotaxis they produce seems to be much more cell-type specific (Bagglioni et al, 1989, Sherry and Cerami, 1991, and Strieter et al, 1994). The group is divisible into two subgroups on the basis of whether they have pairs of cysteine residues separated by a further amino acid (the C-X-C chemokines) or whether the cysteine residues are contiguous (the C-C chemokines). IL-8 was the first of the former to be characterised, whilst MCP-1 was the first of the latter. Although many additions have been made to each family since, it remains generally true that the C-X-C chemokines have a chemotactic influence predominantly on neutrophils whereas C-C chemokines predominantly influence mononuclear cells.

It is therefore of great interest to ask whether IL-8 is involved in the huge neutrophil recruitment so characteristic of pneumonia.

1.15 Interleukin-8

1.15.1 Sources of interleukin 8

IL-8 was first described as a monocyte product (Yoshimura et al, 1987). Macrophages can be induced to produce IL-8 by LPS from gram negative bacteria (Sylvester et al, 1990) and by other inflammatory mediators such as IL-1 and TNFα (Matsushima et al, 1988). Lipotechoic acid from gram positive bacteria can induce IL-8 production from monocytes cells and is likely to induce its expression by alveolar macrophages too (Standiford et al, 1994). Within the lung it can be produced not only by macrophages, but also by epithelial cells (Abdelaziz et al, 1995), fibroblasts (Rolfe et al, 1991) and endothelial cells (Gran et al, 1996). These resident cells do not respond directly to LPS to produce IL-8, but produce it in response to IL-1β and TNFα. Thus they can be regarded as taking part in a network in which the macrophage exerts primary control.

Neutrophils are also a significant source of IL-8 (Bazzoni et al, 1991). Despite producing a significant increase in IL-8 mRNA in response to LPS, they release very little protein, but during active phagocytosis, they release significant IL-8. Although they can produce less IL-8 than macrophages per cell, their huge number at inflammatory foci means they may make a very significant contribution to the total IL-8 at such sites.

Tracheal superfusion with Pseudomonas aeruginosa supernatant produced a neutrophil influx and increase in IL-8, both of which could be blocked by the CD18 inhibitor NPC 15669 (Inoue et al, 1995), suggesting that the IL-8 was neutrophil derived. Migration of neutrophils alone does not seem to activate neutrophils sufficiently for IL-8 release. If IL-8 itself was added to the perfusate instead of Pseudomonas aeruginosa supernatant then neutrophil migration occurred but recovered neutrophils did not show any increase in IL-8 mRNA.

1.15.2 Receptors for IL-8

Two functional neutrophil receptors for IL-8 have been discovered, termed IL-8R A and B. IL-8RA is specific for IL-8 whereas other chemokines such as gro act on IL-8RB. IL-8RB is downregulated during chemotaxis. Both are downregulated rapidly in the presence of exogenous IL-8, but levels of IL-8RA recover more rapidly. This has led to the suggestion that the former may be particularly important at sites of inflammation where levels of IL-8 are high, whereas the latter might be more important in chemotaxis (Chuntharapai and Kim, 1995). These receptors are themselves subject to control of expression, LPS decreasing and G-CSF increasing mRNA levels and ^{125}I-IL-8 binding levels (Lloyd et al, 1995).

In addition, there is a receptor on the erythrocyte which appears to be the Duffy blood group antigen. This is a promiscuous receptor that may bind a variety of different members of the chemokine family (Horuk et al, 1994). It is of particular interest as the erythrocyte receptor for malaria (Horuk et al, 1993).The function of this receptor is not clear. It

presumably acts to buffer the amount of free IL-8 within the plasma. To date, there have been no reports linking the lack of the Duffy blood group to any pathology related to impaired control or use of IL-8.

The specific binding of IL-8 to endothelium has also been described (Rot et al, 1996). Here it may be presented to bound neutrophils to activate them in a similar fashion to that described for PAF (Zimmerman et al, 1992). Rot et al found that there were specific binding sites for IL-8 on post-capillary venules in the skin, but were unable to demonstrate binding to pulmonary vascular endothelium. They also found that no endothelial cells tested in culture (including HUVEC and primary dermal endothelial cultures) expressed binding sites.

This may be important in the interpretation of *in vitro* experiments or extrapolation of experimental observations from one tissue to another.

1.15.3 Actions in vitro

IL-8 causes migration of neutrophils across naked filters in the classical assay for chemotaxis (Kurdowska et al, 1995). At higher concentrations, it also functionally activates neutrophils, causing activation of the respiratory burst (Walz et al, 1991) degranulation (Kurdowska et al, 1995), loss of L-selectin and upregulation of CD18 (Luscinskas et al, 1992) , and increased phagocytosis (Detmers et al, 1991). Chemotaxis to IL-8 may be inhibited by endotoxin from a variety of bacteria (Bignold et al, 1991).

When a filter coated with unstimulated endothelial or epithelial cells is used, IL-8 also induces neutrophil migration (Smart and Casale, 1993). The duration of the response is greater than across naked filters. Anti-IL-8 antibody blocks the chemotaxis at any stage, showing that the prolonged migration is not due to secondary intermediary generation. Presumably this is due to binding of IL-8 and its continued presentation to the neutrophils,

though it should be remembered that cellular IL-8 binding sites have not been shown on cultured endothelial cells (Rot et al, 1996).

IL-8 binds to basement membranes. Basement membrane on which confluent epithelial monolayers had been grown supported migration of neutrophils when an exogenous gradient was applied, but not alone (McCormick et al, 1995). However, if epithelial cells were exposed to Salmonella typhimurium before harvesting the basement membrane, this supported neutrophil flux in the absence of an externally applied gradient. This 'imprinting' of the basement membrane paralleled epithelial IL-8 release, could be blocked by anti-IL-8 antibodies, and was resistant to washing. This suggests that the membrane had tightly bound IL-8 which was able to act as a gradient, and might explain how signalling gradients are maintained *in vivo* despite volume flux.

Despite the ability of IL-8 to induce neutrophil migration through unstimulated endothelial monolayers, it has been found to inhibit the adhesion of neutrophils to activated monolayers (Hébert et al, 1990). This potentially anti-inflammatory action seems at least at first sight surprising given all IL-8's other documented pro-inflammatory effects. It was named the leukocyte adhesion inhibitory (LAI) effect.

Further investigation showed that the LAI effect was shared with FMLP and C5a, but not with GM-CSF, LT B4 or PAF (Luscinskas et al, 1992). All these agents cause L-selectin shedding and CD18 upregulation, so this effect is not just due to shedding of L-selectin on neutrophil activation. In addition, IL-8, FMLP, and C5a were found to cause detachment of neutrophils already adherent to activated endothelial monolayers and to inhibit transmigration across them.

Confocal microscopy demonstrates a rearrangement of F-actin during the LAI effect (Westlin et al, 1992). Treatment of neutrophils with cytochalasin B, which inhibits F-actin formation, inhibits the LAI effect. Clearly it is an active process, and it is reasonable to speculate that it plays a rôle *in vivo*.

In summary, IL-8 is a peptide which can be expressed by a wide variety of cells within the lung (and elsewhere), which causes neutrophil chemotaxis and neutrophil activation. *In vitro,* it can also be demonstrated to have some potentially anti-inflammatory activity by way of inhibiting neutrophil adhesion to activated endothelial cells.

1.15.4 In vivo *actions of IL-8*

Injection of IL-8 into the skin of humans and rabbits causes neutrophil recruitment (Leonard et al, 1991, and Hechtman et al, 1991), as does injection into the pleural space (Utsomiya et al, 1996). The latter showed that co-administration of actinomycin D did not inhibit IL-8-induced neutrophil influx (though it inhibited influx due to TNFα and IL-1β), showing that IL-8 did not work by induction of further mediators. Although Rebeiro et al, 1991 et al found that IL-8-induced neutrophil influx was macrophage dependent in the peritoneum and in subcutaneous airsacs, this study was performed in rats, which have no direct IL-8 homologue.

Endo et al, 1991, found that IL-8 injection into the joint space produced a full inflammatory response, with a mononuclear influx following an initial neutrophil influx. This would not be directly predicted from the *in vitro* actions of IL-8. It may be due to secondary inflammation provoked by neutrophil-induced bystander damage, thus triggering normal inflammatory cascades.

Hechtman et al, 1991 also showed that neutrophil influx due to intradermal IL-8 could be inhibited by simultaneous intravenous IL-8. This may be an *in vivo* manifestation of the leukocyte adhesion inhibitory (LAI) effect. This apparently anti-inflammatory action has also been seen in transfected mice over-expressing IL-8, in whom neutrophil influx to inflammatory sites is diminished (Simonet et al, 1994), and in cardiac ischaemia/reperfusion, when intravenous IL-8 protects against neutrophil influx and damage (Lefer et al, 1991).

The levels of IL-8 have been found to be elevated in a large number of diseases (see table 1-1), both in the lung and elsewhere.

TABLE 1-1 Examples of disease states in which IL-8 is elevated

Extra-pulmonary;
Psoriasis in the skin (Kulke et al, 1996),
Behçet's disease in plasma (al-Dalaan et al, 1995)
Post-cardiac transplant in the coronary sinus (Oz et al, 1995),
During cardiopulmonary bypass in plasma (Kawahito et al, 1995)
Osteomyelitis locally in bone and in the plasma (Klosterhalfen et al, 1996)
Pulmonary;
BAL from idiopathic pulmonary fibrosis (Nakamura et al, 1995),
BAL from hypersensitivity pneumonitis (Sugiama et al, 1995)
Induced sputum from asthma and chronic bronchitis (Keatings et al, 1996)
BAL from the affected lung (but not the unaffected lung or plasma) in community acquired pneumonia (Boutten et al, 1996)
BAL from pneumonia and ARDS (Chollet-Martin et al, 1993).

The macrophage has been shown to be a source of abnormally high IL-8 in a number of circumstances (see table 1-2).

Table 1-2 Diseases in which abnormally elevated macrophage IL-8 production has been shown

Disease	*Reference*
Rheumatoid arthritis	Endo et al, 1991
Cystic fibrosis	Strieter et al, 1993
Pulmonary fibrosis	Carre et al, 1991
Inflammatory bowel disease	Grimm et al, 1996

This list of conditions in which elevated levels have been found is by no means comprehensive. However, the mere demonstration of high levels tells us little of the functional rôles of IL-8. In many of these conditions one would expect the levels of other cytokines and inflammatory mediators to be elevated too. Although to prove a role for IL-8 in a given condition it must be possible to demonstrate its presence, the amount of IL-8 present will say little about its functional importance compared to these other mediators.

The finding that IL-8 levels correlate with neutrophil numbers in synovial fluid (Endo et al, 1991), broncho-alveolar lavage fluid of patients with ARDS and pneumocysitis

pneumonia (Villard et al, 1995) and sputum sol phase in asthma and bronchitis (Nocher et al, 1996) must be interpreted with caution. As neutrophils themselves produce IL-8, elevated IL-8 levels could be the result rather than the cause of increased neutrophil numbers. More convincing is the demonstration that macrophage IL-8 mRNA correlates with neutrophil numbers in pulmonary fibrosis (Carre et al, 1991), and that in Hodgkin's disease, there is a topographical relationship between macrophage IL-8 production and neutrophil influx (Foss et al, 1996). On the other hand, the lack of such a correlation in skin blister fluid (Follin et al, 1991) and bacterial pneumonia (Villard et al, 1995) does not necessarily argue against an important rôle for IL-8 in neutrophil influx. Even if neutrophil clearance remains constant, the neutrophils seen to have accumulated will have done so over a period of time, during which IL-8 levels could have been radically different than those measured at a single point.

More usefully, the ability of anti-IL-8 antibodies to inhibit chemotactic activity of biological fluids has been assessed (see table 1-3). Inhibition has been between 27 % and 75 %, suggesting that although IL-8 contributes to chemotactic activity in these states, other factors are at least as important.

Table 1-3 Inhibition of chemotactic activity in biological fluids by anti-IL-8 antibody

Fluid (disease state)	*Inhibition (%)*	*Reference*
Synovial fluid (rheumatoid arthritis)	27-57	Koch et al, 1991
Pleural fluid (LPS induced pleurisy)	65-75	Brylan et al, 1994
Pleural fluid (empyaema, parapneumonic effusion)	30	Antomy et al, 1993
BAL (Cystic fibrosis)	30	Strieter et al, 1993
Tracheal superfusate (Post antigen exposure)	75	Kaneko et al, 1996

IL-8 has also been correlated with disease severity in ARDS (Groenweld et al, 1995) and cystic fibrosis (Strieter et al, 1993). In a group at risk of developing ARDS, very early elevated broncho-alveolar lavage IL-8 levels predicted those who would progress to disease (Donnelly et al, 1993). This suggests a causal relationship.

The most convincing data suggesting an important rôle for IL-8 in neutrophil recruitment comes from antibody inhibition studies. Anti-IL-8 antibody inhibits neutrophil influx after the addition of Staph. aureus to tracheal superfusates (Masson et al, 1995), LPS-induced pleurisy (Broaddus et al, 1994), acid aspiration (Folkesson et al, 1995) and immune-complex mediated glomerulonephritis, LPS and IL-1 induced arthritis, and lung reperfusion injury (Harada et al, 1994). All these studies measured total neutrophil accumulation, so did not measure influx specifically. In addition, they all administered antibody prior to the inflammatory stimulus (with the exception that in some experiments by Folkesson et al, 1995, antibody was administered 1 hour after). Thus they give no information on the relative importance of IL-8 as the inflammatory response evolves.

This has only been addressed by Collins et al, 1991. After intraperitoneal zymosan, peritoneal fluid was injected into rabbit dorsal skin and assayed for the ability to cause neutrophil recruitment. This *in vivo* chemotactic assay showed that within two hours, complement contributed most to neutrophil recruitment, but later, IL-8 and another similar molecule (later identified as gro Jose et al, 1991) were pre-eminent.

In summary, IL-8 causes neutrophil recruitment when administered to tissues, but may inhibit it when administered intravenously. High levels have been associated with a number of disease processes, and sometimes this correlates with neutrophil numbers. The chemotactic activity of biological fluids from inflamed sites has chemotactic activity that can be inhibited only partially by anti-IL-8 antibody. Anti-IL-8 antibody generally inhibits neutrophil accumulation, but these experimental protocols have not determined whether the rôle of IL-8 varies with time. The one study that addresses this suggests that IL-8 is not always equally important throughout the inflammatory process.

1.16 The clearance of neutrophils from inflammatory sites

Whilst there has been a huge interest over the past century and a half in the mechanisms of neutrophil recruitment to inflammatory sites, the mechanisms by which the vast numbers of cells which accumulate are subsequently removed has been very little studied until recently. In a three volume work reviewing experimental study of the inflammatory process published in 1974 , the only specific discussion of neutrophil clearance is a reference to the fact that 10^{11} neutrophils must be removed from the body per day during normal turnover (Zweifach et al, 1974). It was considered that the majority were lost into the gastrointestinal tract and from mucous membranes, but no consideration was given to the fate of the neutrophils once in the tissues.

Others have acknowledged the problem. Heffron in his extensive monograph on pneumococcal pneumonia suggested that there must be an active process of 'absorption', though he was unable to elaborate on the mechanisms that brought this about (Heffron, 1939). As neutrophils have never been observed to leave the tissues following their migration there from the blood, it has often been presumed that they must passively degenerate and be cleared by scavenging macrophages (Hurley, 1983, and Sanui et al, 1982).

Such neutrophil engulfment by macrophages was seen in early studies of pneumonia. Loosli in observations on primates noted;

"the polymorphonuclear leukocytes appeared early and in large numbers, but they began to degenerate and to be taken up by the mononuclear macrophages after 24 hours"
(Loosli, 1942a)

In fact, in an accompanying paper on pneumonia in the dog, he went on to observe of the neutrophils that:

"although they were the most numerous cell type in the exudate up to 48 hours, many at this time had pycnotic (sic) nuclei and had been taken up by the mononuclear macrophages"
(Loosli, 1942b)

Although it was not realised at the time, in retrospect Loosli's description of neutrophils with pyknotic nuclei being phagocytosed by macrophages was one of the earliest descriptions of a specific mode of cellular death that remained uncharacterised for more than 30 years, and undescribed in the neutrophil for nearly half a century.

Neutrophils are known to contain a vast array of potentially histotoxic compounds. If neutrophils were to die passively at the site of inflammation, these would presumably be released. This would mean that acute inflammation would inevitably be associated with uncontrolled tissue damage, which might be regarded as a design fault in the system! It would also be likely to lead to further secondary generation of pro-inflammatory mediators. It is now known that cells in general die by one of two mechanisms; not just by passive necrosis but also by the far more controlled process of apoptosis (programmed cell death).

1.16.1 Apoptosis and necrosis

The term apoptosis was first used by Kerr and colleagues (Kerr et al, 1972). It is derived from a Greek word used to describe the falling of leaves from trees in the autumn, and was chosen as an expression of a natural mechanism of death as part of an ongoing life-cycle.

It is now generally accepted that cell death occurs either by necrosis or by apoptosis, the morphological features of which have been extensively reviewed (Kerr et al, 1987, Wyllie et al, 1980). Necrosis is usually seen as a response to an unusual pathological process such as ischaemia or trauma. Confluent cells are often affected, with an associated secondary inflammation. Individual cells swell, as do their mitochondria. Ultrastructurally, there may be some evidence of nuclear pyknosis but this is not prominent or clearly defined. Membrane integrity is lost early, giving rise to inability to exclude vital dyes.

Apoptosis is usually seen in irregularly distributed isolated cells. There is contraction of the cell with surface blebbing, an active process that has been named zeiosis. The cell

membrane retains its integrity, excluding vital dyes. The cytoplasm shows vacuolation without the gross mitochondrial dilation seen in necrosis. Indeed, the cell's cytoplasmic organelles characteristically are maintained intact. The chromatin undergoes condensation and contracts to form dense clumps around the nuclear membrane. There is then often fragmentation of the entire nucleus. *In vivo* these are taken up by macrophages, but if this does not occur (e.g. in cell culture) secondary degeneration occurs with swelling and lysis of the cells. Biochemically, the neutrophil breakdown is associated with inter-nucleosomal chromatin cleavage, giving a characteristic 'step-ladder' appearance on DNA gel electrophoresis. The description of apoptosis leading to nuclear pyknosis with subsequent uptake of the apoptotic cells by surrounding macrophages appears to be exactly Loosli's description of cells in pneumonia in 1942.

1.16.2 Apoptosis of the neutrophil and its recognition by macrophages

A number of reports of phagocytosis of neutrophils by macrophages have appeared over the past 30 years. Elicited mixed leukocytes from the guinea pig peritoneum mixed with Staphylococci produced such examples on electron-microscopy (Brewer, 1964). Later it was demonstrated that such phagocytosis was associated with aged neutrophils, implying a specific change in them allowing them to be recognised as senescent cells by the macrophages (Newman et al, 1982). Later, the morphological changes of the neutrophil as it ages were recognised to be those of apoptosis (Savill et al, 1989a). This was not mediated by the macrophage receptor associated with uptake of senescent erythrocytes or by Fc receptors, but by a novel charge-sensitive mechanism (Savill et al, 1989b). It was also found to be inhibited by antibodies to the vitronectin receptor, the $\alpha_v\beta_3$ integrin (Savill et al, 1990). Additionally, antibodies to the thrombospondin receptor, CD36, also blocked the recognition of aged neutrophils. Thrombospondin is produced by macrophages and was shown to facilitate the interaction when added exogenously, and to adhere to neutrophils. It

was hypothesised that thrombospondin forms a molecular bridge between CD36 and the vitronectin receptor on the macrophage and an as yet unknown receptor on the neutrophil surface that appeared as apoptosis took place.

The original work describing the importance of the thrombospondin and vitronectin receptor was performed using human peripheral blood monocytes, aged for 4 days *in vitro*. These cells gain a macrophage phenotype. Murine bone marrow-derived macrophages were found to use similar recognition mechanisms. However, inflammatory macrophages elicited by protease peptone or thioglycollate broth injection into the peritoneal cavity were able to ingest apoptotic neutrophils by a different mechanism (Fadok et al, 1992). This was inhibitable by phosphatidylserine, a membrane phospholipid that is usually confined to the internal aspect of the membrane, but has been found to be externalised in apoptotic lymphocytes. Thus the process of recognition and uptake of apoptotic neutrophils is different at different sites. It may even be that different mechanism are used in normal physiological homeostasis and pathological inflammation.

Although macrophage uptake of senescent neutrophils has correlated well with morphological apoptosis in these experiments, changes leading to recognition and to DNA cleavage are not necessarily directly related in sequential fashion, but may normally proceed in parallel. That the two may be dissociated is demonstrated by mice transfected with the bcl-2 oncogene (Lagasse and Weissman, 1994). Normally bcl-2 expression is lost in mature neutrophils. In these mice it continued to be expressed. They showed diminished apoptosis, but normal neutrophil kinetics with continued macrophage ingestion of neutrophils.

Neutrophil apoptosis differs from apoptosis in many other cells studied in that it is accelerated by protein synthesis inhibition by cyloheximide (Payne et al, 1994). However, other ATP/ADP ratios are preserved within the apoptotic neutrophil, whilst their glutamine and lactate decrease, suggesting that basic cellular respiration continues (Nunn et al, 1996).

It is suggested that normally a protein is produced that inhibits apoptosis and removal of this protein is the triggering factor for apoptosis.

During the process of apoptosis, many neutrophil functions are altered. In response to FMLP there is decreased shape change, chemotaxis, degranulation, respiratory burst and phagocytosis of opsonised zymosan. Although this was partially due to loss of the FMLP receptor, this was not the entire explanation, as superoxide production was preserved in response to PMA (Whyte et al, 1993a).

The adhesion molecules also show significant changes. L-selectin expression is decreased, whilst that of CD18 integrins is increased. Adherence to E-selectin is decreased, as is adhesion to fibrinogen, the latter demonstrating that the increased surface CD18 molecules are functionally inactive (Dransfield et al, 1995). Surface expression of the FcγRIII (CD16) molecule is also decreased on apoptotic cells (Dransfield et al, 1994).

All these processes conspire to make the apoptotic neutrophil relatively quiescent and isolated from potential activating factors in their environment. In addition, when they are phagocytosed by macrophages this does not lead to secondary activation of the macrophage, unlike other forms of macrophage phagocytosis (Meagher et al, 1989). The whole process of neutrophil apoptosis and uptake by macrophages as demonstrated *in vitro* thus has huge potential to act as an injury-limiting mechanism for neutrophil clearance.

Teleologically speaking it is easy to see that modulation of neutrophil apoptosis and uptake by macrophages would be beneficial. Neutrophils recruited to an inflammatory focus must persist long enough to perform their protective function, which could mean prolongation of their life beyond that usually seen in the peripheral circulation. Having completed this task, it might then be beneficial to promote apoptosis, rendering the neutrophils relatively quiescent and preventing secondary damage, and promoting their clearance by macrophages. There is abundant *in vitro* evidence of the facility for just such a modulation to take place.

1.16.3 Modulation of neutrophil apoptosis and its recognition

Many neutrophil activating agents act by raising intracellular calcium. Such an elevation causes decreased neutrophil apoptosis, whereas chelation of intracellular calcium increases apoptosis, opposite to the effects witnessed in many other cell types (Whyte et al, 1993b). This would lead one to expect that neutrophil activating agents would tend to decrease apoptosis, which is indeed what is generally found. This would be consistent with a prolongation of neutrophil functional activity at an inflammatory site where the concentration of mediators would be elevated.

Conditioned medium from monocyte and bronchial epithelial cell cultures reduce apoptosis by the generation of GM-CSF and G- and GM-CSF respectively (Lee and Haslett, 1994, and Cox et al, 1992). Neutrophils were taken from patients with paediatric malignancies treated with G-CSF *in vivo,* and aged *in vitro*. There, they too showed decreased apoptosis compared to controls (Adachi et al, 1994).

Similar effects were also obtained with pure preparations of these cytokines (Colotta et al, 1992). This latter study also demonstrated that apoptosis was reduced by IFN-γ, LPS and inactivated Streptococci. However, they found no effect of FMLP, C5a and IL-8.

Others have found that C5a and FMLP inhibit apoptosis (Haslett, 1992 and Lee et al, 1993). This is certainly consistent with the same group's findings on intracellular calcium concentrations. Apparent disagreement over the effect of IL-6 was resolved when it was shown that IL-6 indirectly decreases apoptosis by inducing PAF. In experimental conditions, with high neutrophil concentrations, sufficient PAF concentrations are induced by IL-6 to produce clear effects on apoptosis, whereas, when neutrophils are more dispersed, the increase is insufficient to give a clear effect (Biffl et al, 1996). Differences in second mediator generation by neutrophils cultured in different conditions could account for the differing observations on chemoattractants, though similar culture media and cell concentrations were used by each group. Haslett's group cultured the cells in suspension in

teflon bags, whereas Colotta et al cultured neutrophils in flat bottomed culture plates, which may have provided an additional adhesion signal that affected the response. IL-2 also delays apoptosis (Pericle et al, 1994).

ATP and other adenosine products that may be released by activated platelets decrease neutrophil apoptosis (Gasmi et al, 1996). This may be important as platelets adhere to damaged endothelium at inflammatory sites and release mediators.

Glucocorticoids inhibit neutrophil apoptosis. This is particularly interesting as they have the opposite effect on eosinophils, closely related members of the granulocyte lineage (Meagher et al, 1996). This indicates the high degree of specificity of the control mechanisms.

During pneumonia there is likely to be a significant degree of hypoxia in the lung, particularly at sites with a dense cellular infiltrate. Hypoxia has been shown to decrease apoptosis, an effect that could be mimicked by catalase but not by superoxide dismutase, Trolox or methionine (Hannah et al, 1995).

Conversely, oxidant stress caused by pharmacological depletion of GSH causes an increase in apoptosis that is inhibitable by N-acetylcyseine (Watson et al, 1996). This could be important in providing a feedback control of inflammation in areas exposed to high local reactive oxygen intermediaries generated by local inflammatory changes, and is one of the few stimuli shown to enhance neutrophil apoptosis.

TNFα is the best known agent capable of enhancing neutrophil apoptosis (Takeda et al, 1993). In fact, subsequent studies have shown this to be rather more complex. Early neutrophil apoptosis (4 - 8 hours) is stimulated, but at late time points (21 hours) there is actually an inhibition of apoptosis (Murray et al, 1997).

Fas ligand works through Fas (CD95), a member of the TNF receptor family. Fas is expressed by the neutrophil, and Fas ligand is also released by neutrophils, the only granulocyte to do so. Activating anti-Fas antibody induces neutrophil apoptosis, and Fas-

IgG chimeras partially suppress constitutive neutrophil apoptosis *in vitro*, suggesting that it may have a particularly important rôle to play (Liles et al, 1996). The *in vivo* expression of Fas and Fas ligand during acute inflammation has yet to be studied in great detail.

A variety of proteases (pronase, chymotrypsin and trypsin) induce neutrophil apoptosis *in vitro* (Trevani et al, 1996). This provides a possible negative feedback control during inflammation. If there were excessive inflammation, active proteases might well increase. These would then feed back on the neutrophil to induce the refractory state of apoptosis, limiting further protease release.

Interestingly, erythromycin (and not other antibiotics) has been found to enhance apoptosis (Aoshiba et al, 1995). It is also of unique utility in diffuse panbronchiolitis, a rare inflammatory disease of the airways confined to the Far East. It is interesting to speculate that erythromycin's efficacy could be due not only to its antibiotic but also to its anti-inflammatory actions by way of accelerating apoptosis. These workers correlated erythromycin's actions with an increase in cAMP, which contradicts the extensive pharmacological manipulations performed by others who correlated an increase in cAMP with a decrease in apoptosis (Rossi et al, 1995). The reason for the discrepancy is not apparent. The former group used electronmicrosopy to score apoptosis at 24 hours in culture, whereas the latter used CD16 expression at 21 hours, but it seems surprising that two studies should produce opposite conclusions on the basis of different scoring techniques.

The modulation of neutrophil uptake by macrophages has also been examined. GM-CSF, IFNγ, IL-1β, TNFα, and TGFβ1 all enhance the uptake of apoptotic neutrophils when added to macrophages in culture, both in terms of the proportion of macrophages ingesting cells and the number of neutrophils ingested per macrophage (Ren and Savill, 1995). Pre-treatment experiments established that mediators exerted their effect on the macrophage rather than on the neutrophil. Although the uptake appeared to be via the

thombospondin/vitronectin receptor mediated pathway, no changes in the expression of either receptor or thrombospondin expression was observed. A change in the functional status of the receptors was therefore hypothesised.

There is thus abundant evidence that neutrophil survival can be modulated by a wide range of factors *in vitro*, and that the majority of inflammatory mediators inhibit apoptosis and lengthen life-span in the neutrophil. Uptake of apoptotic neutrophils by macrophages can also be modulated by inflammatory mediators.

1.16.4 ***Evidence for apoptosis*** *in vivo*

Historical studies can in retrospect be seen to have been describing neutrophil apoptosis and clearance by macrophages. The engulfment of neutrophils was described by Metchnikoff in the 19th Century (see Savill, 1992). The work of Loosli describing 'degeneration' of neutrophils with pyknotic nuclei which are taken up by macrophages during pneumonia has been described above (Loosli, 1942b).

Neutrophils phagocytosed by macrophages were found to be quite prominent in joints inflamed by Reiter's syndrome (Perkin et al, 1966). Figures produced in these papers show engulfed neutrophils with condensed cytoplasm, with other neutrophil in varying states of degeneration.

A resected spleen from a patient with thrombotic thrombocytopaenic purpura showed that neutrophils (as well as other blood cells) had been engulfed by macrophages (Kadri et al, 1975).

Macrophage phagocytosed neutrophils were also described in childhood chronic benign neutropaenia (Parmley et al, 1981). In this case, neutrophil band forms were phagocytosed in addition to older neutrophils. Some of the plates produced appear to show the phagocytosis of non-apoptotic neutrophils. The process described in this disease seems unusual, and one wonders if a defect in the normal recognition process (for instance

neutrophils expressing surface changes normally associated with ageing early in their life-span) contributed to the disorder, but as apoptosis was not widely appreciated at the time (and had not been described in granulocytes) this was not investigated by the authors.

The injection of a number of stimuli into guinea-pig peritoneum was followed after an interval by an increase in the number of macrophages showing evidence of ingested neutrophils. In some cases as many as 20 % of macrophages showed evidence of neutrophil ingestion (Sanui et al, 1982). The timing of the response depended on the initial stimulus, with thioglycollate broth or oyster medium producing a peak in 48 hours, whereas Listeria monocytogenes and Staphylococcus aureus produced a peak after 72 hours.

Full appreciation of the importance of neutrophil apoptosis is difficult *in vivo* because on histological sections it is very difficult to detect. This is because the interval between morphological apoptosis and uptake by surrounding macrophages is short. It has been estimated that this period is usually of the order of 30 minutes. Apoptotic bodies may, for a longer, period be recognisable within the macrophage but breakdown is rapid, so this interval is of the order of a few hours at most (Ellis et al, 1991, Kerr et al, 1987, and Wyllie et al, 1980). Thus apoptosis can lead to a huge reduction in cell numbers over a period of time, but in static morphological images it will appear a rare event. In a study in which mice were administered anti-CD4 antibodies, it was found that apoptosis was caused leading to loss of 50 % of T cells over 48 hours (Howie et al, 1994). This was associated with a maximum of 1.33 % unequivocally apoptotic neutrophils at any one time, compared to 0.55 % in controls. Thus, very small changes in the visible rate of apoptosis may have profound effects on overall cell kinetics.

Despite these difficulties, a number of studies have been performed which suggest that the process is important *in vivo*. A histological study of the marrow of normal neonatal mouse bone marrow showed significant amounts of apoptotic neutrophils, with uptake by marrow macrophages (Sasaki et al, 1995).

Labelling of rabbit donor neutrophils with BrdU allowed their tracking when injected into donor animals. After several hours they became predominantly localised within the spleen. Extraction of DNA showed the BrdU-labelled neutrophil DNA to have been cleaved in the inter-nucleosomal pattern characteristic of apoptosis (Bicknell et al, 1994). This suggests that apoptosis is the normal limiting factor in the life span of the circulating neutrophil.

Between 1 % and 11 % of neutrophils from inflamed synovial fluid were morphologically apoptotic, and synovial macrophages were observed to contain apoptotic neutrophils (Savill et al, 1989). Apoptotic neutrophils were also observed in broncho-alveolar lavage fluid from neonates with respiratory distress syndrome (Grigg et al, 1991).

Two abstracts were presented to the American Thoracic Society describing the appearance of apoptotic neutrophils in the lung. In the first of these, approximately 2 % of neutrophils from patients with ARDS were apoptotic morphologically (Matute-Bello et al, 1996). Surprisingly the authors found that only 2 % of all apoptotic neutrophils were within macrophages.

In the second abstract, the presence of apoptotic neutrophils in pneumococcal pneumonia is examined (Kutkoski et al, 1996). Here, the process of *in situ* end-labelling was used. This technique uses the characteristic double-stranded DNA breaks as a template for use of terminal transferase to add a label (Gold et al, 1994). (DNA breaks in early necrosis tend to be single stranded and thus not recognised by the transferase). This technique raises some questions as it is highly dependent on the phase of apoptosis or necrosis, the rapidity of fixation to prevent further DNA digestion post-mortem, and the degree of Proteinase K pre-treatment. Whether it is entirely applicable to intense inflammatory conditions is open to some doubt. Using this technique it was found that as many as 50 % of neutrophils in the lung were apoptotic. Fewer cells recovered by broncho-alveolar lavage were apoptotic, but these were assessed by more conventional morphological criteria, and numbers were not

specified in the abstract. Furthermore, it would be expected that more cells would exhibit apoptosis if assessed at an early molecular level rather than at a stage late enough to permit identification by gross morphological change.

Injection of thioglycollate broth into guinea pig peritoneum produces a neutrophilic inflammation (Yamamoto et al, 1993). After a delay, an increase in the number of neutrophils observed within macrophages is seen as they are cleared following apoptosis. Co-injection with LPS delayed this peak by 24 hours, and neutrophil numbers were elevated. It was suggested that this shows the *in vitro* delay in apoptosis produced by LPS was being reflected *in vivo.* This is complicated by the fact that the LPS itself will produce an inflammatory action, so the change could be interpreted as being secondary to a larger initial inflammatory stimulus. This was not controlled for, so the results must be interpreted with caution.

LPS instillation into the lungs of rabbits produced a brisk inflammatory reaction. In broncho-alveolar lavage there was found to be an initial peak in neutrophils at around 18 hours, followed by an increase in the number of apoptotic neutrophils at around 24 hours, which coincided with an increase in the number of alveolar macrophages containing apoptotic bodies (Cox et al, 1995). This strongly suggests that the *in vitro* data demonstrating neutrophil apoptosis and macrophage uptake is relevant *in vivo.*

As reviewed above, the majority of pro-inflammatory mediators studied appear to have an inhibitory influence on apoptosis. Despite this, synovial fluid taken from inflamed human joints was added to neutrophil cultures and found to increase rather decrease apoptosis, an effect unrelated to the levels of G- and GM-CSF in the fluid (Bell et al, 1995). This was true of neutrophils derived either from the peripheral blood or from the inflamed joints themselves. The mechanism of this effect is unclear, but it does certainly seem clear that factors present at inflammatory sites are capable of modulating apoptosis.

A combination of *in vitro* and *in vivo* techniques was used to further study the relevance of *in vitro* findings to neutrophil apoptosis *in vivo*. Neutrophil ageing was studied using neutrophils obtained from rats in a variety of different circumstances. Peripheral blood neutrophils from normal rats were compared with bone marrow derived neutrophils, peripheral blood neutrophils from animals during peritonitis induced by protease peptone, or inflammatory neutrophils from the peritoneum 7 or 12 hours after induction of peritonitis. Neutrophil apoptosis after 12 hours *in vitro* was inhibited in neutrophils taken from peripheral blood during inflammation, and in neutrophils taken from the inflammatory site 12 hours after the induction of inflammation compared to normal peripheral blood neutrophils. Likewise, marrow neutrophils were relatively resistant to apoptosis. This overall pattern was closely mimicked by the ability of cycloheximide to induce apoptosis. This was greatest in normal neutrophils and relatively inhibited in inflammatory neutrophils. However, the ability of cycloheximide to enhance apoptosis was maintained in bone marrow neutrophils. TNF was able to enhance apoptosis as assessed at 3 and 6 hours in normal peripheral blood neutrophils, but this effect was not seen in other neutrophils, an effect that could not be attributed to change in overall receptor number (Tsuchida et al, 1995). This suggests that the neutrophil's susceptibility to apoptosis may be controlled during normal physiological development and subject to further controls during inflammation.

Taking the last two studies together it is interesting that neutrophils taken from inflammatory environments show different susceptibilities to apoptosis when cultured in similar conditions *in vitro*, whilst identical neutrophils can be altered by culturing them in biological fluids from inflammatory sites, suggesting that the final rate of apoptosis observed is a product of past and current environments of the neutrophil. This suggests a very dynamic control.

In summary, *in vivo* evidence to date is consistent with *in vitro* data showing that ageing neutrophils undergo apoptosis and are then ingested by macrophages. This has been shown to be modulated in complex ways that may be more complex and varied than is immediately apparent from purely *in vitro* results.

1.17 Experimental work

Since the neutrophil is clearly a critical cell in pneumonia, experiments to investigate its behaviour further were performed. These were planned with particular considerations as follows:

1) Although the neutrophil is important in all forms of acute inflammation, the unique structural and functional relationships of the neutrophil and the pulmonary circulation means that lessons learned from inflammation affecting other areas in the body cannot necessarily be directly applied to inflammation in the lung.

2) Inflammation is an ongoing, evolving process. It is therefore appropriate to investigate it at different time points in its evolution, as the same mechanism utilised during early induction of inflammation (the first few hours having received the overwhelming focus of attention in the past) are not necessarily the same as those utilised at a later stage. This requires accurate timing of the onset of pneumonia. This makes an animal model of the disease invaluable, as the timing of onset in humans would be extraordinarily difficult to establish.

3) As neutrophil clearance as well as recruitment appears to be an active process capable of specific modulation, mere counting of neutrophils within an inflammatory site will give limited mechanistic information. Numbers will depend on the integral with time of the difference between the rate of influx and the rate of efflux of neutrophils. A change in either parameter may be the cause of a change in total number. Indeed, if these parameters were to alter simultaneously, misleading conclusions could easily be drawn. If, for example, there were a large increase in clearance at the same time as a small increase in influx, total neutrophil numbers would fall. In most studies to date, this would have been regarded as evidence of decreased influx. It is clearly important to use techniques capable of resolving the two processes if at all possible.

4) As already discussed, pneumonia may be divided into community acquired and nosocomial pneumonia, each of which have different characteristic aetiologies, pathologies, and prognoses. There is ample evidence that the inflammatory process shows great specificity, so it is valuable to use models of each type of disease. In particular, it was noted that nosocomial gram negative pneumonia is characteristically associated with lung damage, whereas community acquired pneumococcal pneumonia is notable for the efficiency of restoration of lung architecture. In view of the fact that neutrophils may be responsible not only for host defence but also for bystander host damage it was hypothesised that tissue neutrophil burden might be greater in typical nosocomial pneumonia than in typical community acquired pneumonia, due either to prolonged neutrophil influx, delayed clearance, or a combination of the two. Inclusion of both types of pneumonia in experimental studies allows examination of this overall hypothesis.

With these criteria in mind, a model of pneumonia using the New Zealand White Rabbit was selected. Streptococcus pneumoniae and Escherichia coli were selected as typical examples examples of CAP and NP respectively. Appropriate bacterial suspensions could be simply and accurately placed intrabronchially via a fibre-optic bronchoscope, mimicking the aspiration that is thought to be the usual method of bacterial acquisition during development of pneumonia. This allows the onset of pneumonia to be accurately defined, and produce a localised pneumonia without excessive effect on the animals' general health.

Neutrophils were then used from a donor pool and labelled with ^{111}indium chloride, and injected at specified intervals after induction of pneumonia. Gamma counting (either in a well-counter or by external scintigraphy) could then be used to follow the influx of these cells to the site of the pneumonia. To ensure that specific recruitment was measured rather than just a blood pool effect, autologous ^{99m}technetium-labelled erythrocytes were used to correct for this. Interventions then allowed study of their effect specifically on influx. The

CD18 adhesion molecule and IL-8 were selected for study in view of existing data suggesting important rôles for these two molecules during inflammation.

Further studies were then performed to look for evidence of apoptosis in the clearance of neutrophils and additional *in vitro* experiments were performed to examine whether factors produced *in vivo* might be responsible for modulating neutrophil survival.

The remainder of this thesis describes and discusses experiments performed with this model.

2. STUDIES OF NEUTROPHIL INFLUX

2.1 Introduction

As I have extensively discussed in the introductory chapter, neutrophils are recruited to areas of pneumonia where they provide valuable defence against invading pathogens. Inflammation always sees the paradox that whilst neutrophils perform this important defensive rôle, the array of toxic substances they are capable of producing means that their recruitment always risks bystander damage. It is thus essential that tight control be exercised over their numbers in the tissues.

Pneumonia due to Streptococcus pneumoniae (pneuS) is a typical example of community acquired pneumonia. It is also an archetypal example of beneficial inflammation in that it is usually followed by prompt and complete restoration of normal tissue structure and function. Pneumonia due to Escherichia coli (pneuE) is typical of nosocomial pneumonia. These pnemonias characteristically produce high levels of mortality, and are often associated with lung damage. It is hypothesised that a contributory factor in this is an excess neutrophil load. Either prolonged neutrophil influx, impaired neutrophil clearance, or both may lead to this.

The experiments in this chapter concentrate particularly on defining the time course of neutrophil influx in rabbit models of the two types of disease, to see if the influx pattern differs. A more prolonged neutrophil influx in pneuE may contribute to an increased tissue neutrophil burden, with consequent increase in secondary tissue damage.

2.2 Methods

Chemicals and plastics were obtained from Sigma, Poole, unless otherwise specified.

2.2.1 Rabbits

Specific pathogen free New Zealand White rabbits (Charles River Breeding, Margate) weighing approximately 3 kg were used as experimental subjects, and were housed in accordance with Home Office guidelines. They were allowed at least 2 days recovery post-transit prior to experimentation.

In addition, a colony of rabbits of similar origin were maintained as blood donors, and these grew up to 8 kg. They donated a maximum of 64 ml of blood per session, with a minimum interval of 1 month between donations. Any animal with a haematocrit below 0.43 had its next donation deferred for a further month. Animals showing any sign of ill health were not used.

For blood donation, the rabbit ear was smeared with lignocaine/prilocaine anaesthetic cream (Emla®, Astra Pharmaceuticals, King's Langley). Animals were held in a custom made restrainer, and xylene applied to the ear tip to cause vasodilation. Fur was plucked from over the central ear artery, and a 19G hypodermic needle (Beckton Dickinson UK Ltd., Oxford) was introduced. Blood from this was directed into a 50 ml polypropylene centrifuge tube (Corning, Sigma, Poole) containing 8 ml of 3.8 % sodium citrate solution (Phoenix Pharmaceuticals) to a total volume of 40 ml per tube.

2.2.2 Gamma well counter and gamma camera

Gamma well counting was performed with a 1282 Compugamma CS counter (Wallac). This was used in multiple isotope mode to measure the 140 keV peak of

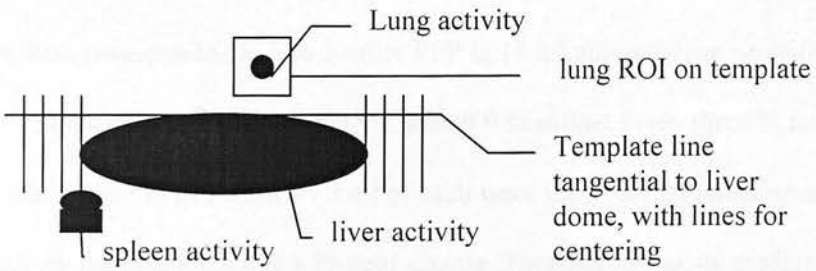
^{99m}technetium and the 247 keV peak of ^{111}indium. Fresh reference samples were used for each batch of counting, with automatic spillover and half-life corrections.

Gamma camera imaging used a Siemens ZLC-3700 camera (Siemens Gammasonics, Inc, Des Plaines, Illinois, USA). A high energy, gallium collimator was used, with dual isotope acquisition using 140 $\pm$ 20 keV and 247 $\pm$ 20 keV windows.

Routine imaging used whole animal posterior, static 300 second images, with animals prone, on a table a fixed distance from the gamma camera head. Data was analysed on-line using dedicated Bartec Unix 4.2 software on a Sun-3/160M workstation. Assessment of lung activity utilised a lung region of interest (ROI) created using the same template in all studies.

Figure 2-1 Construction of lung regions of interest

The figure shows a schematic diagram of a ^{111}indium gamma image, indicating lung, liver and spleen images. Superimposed on this is the template used for drawing lung regions of interest (ROI). The horizontal line is placed tangential to the dome of the liver. Vertical gradations allow this to be centred. A rectangular ROI is then created to match the pre-drawn lung ROI on the template.This was placed tangentially across the dome of the liver and centred, producing a rectangular ROI over the right lung field, clear of the liver.



The count was normalised to 90 pixels, as the resolution used meant, even with the use of the template, slightly different numbers of pixels were included in the ROI. 300 second scans were taken with no source in front of the gamma camera, and these background counts subtracted from controls.

Reference ROI's were created using the ^{99m}technetium image. The largest rectangle possible was constructed over the abdomen, whilst avoiding the liver, spleen, kidneys and midline vessels.

Scintillation counting of rabbit cages and bedding revealed no loss of activity from the rabbits during the experiment. This allowed the whole animal images acquired to be used as measurement of total injected activity. As total activity and region activity were measured simultaneously, the need for half-life correction was eliminated.

2.2.3 Isolation of rabbit neutrophils

The neutrophil isolation procedure was based on that of Haslett et al, 1985a, later modified for rabbit neutrophils (Haslett et al, 1987). This was shown to produce neutrophils with little priming or activation. Blood obtained as described was centrifuged at 300g for 20 minutes at room temperature (using an MSE centrifuge, Fisons, Crawley). The supernatant was aspirated and centrifuged at 2500g for 10 minutes to provide platelet poor plasma (PPP). 0.9 % saline was added to the blood cells to a volume of 50 ml. After mixing, this was allowed to sediment for 30 minutes. The leukocyte-rich supernatant was aspirated from above the sedimented erythrocytes, and centrifuged at 300g for 6 minutes. The sedimented cells were resuspended in 2 to 3 ml of PPP in 15 ml polyethylene centrifuge tubes.

Stock Percoll® was diluted 9:1 with 0.9 % saline. Forty-three % and 53 % solutions of this were made in PPP, and 1 - 2 ml of each were successively underlayered beneath the leukocyte preparation using a Pasteur pipette. This discontinuous gradient was centrifuged for 10 minutes at 275g. Mononuclear cells collected at the upper gradient interface, and were aspirated and discarded. Neutrophils collected around the lower discontinuity, together with some erythrocytes. These were mixed with 10 ml PPP and centrifuged at 275 g for 10 minutes. Erythrocytes in the cell pellet were lysed by the addition of cold 0.2 % saline for 30 seconds, following which isotonicity was restored with an equal volume of 1.6 % saline,

using solutions sterilised by passage through 0.2μm filters. Ten ml of PPP was added before centrifuging the cells at 275g for 6 minutes. Cells were then washed with PPP. An aliquot was counted using a haemocytometer and duplicate 100μl cytocentrifuge preparations stained with Diff-Quik® for assessment of differential cell counts. Viability was assessed by adding 20μl to an equal volume of Trypan Blue. Cells were then assessed in suspension for their ability to exclude the dye.

2.2.4 ^{111}Indium-labelling of neutrophils

To study four rabbits, neutrophils from six tubes (196 ml) of donated blood were used. 8 - 12 MBq of ^{111}indium chloride (Amersham International PLC, Amersham, Bucks.) was added to 50μl of 4mM tropolone (2-hydroxy-2,4,6-cycloheptatrienone) in normal saline. This was allowed to chelate for 2 minutes before addition to the neutrophils, suspended in 40-50 μl of PPP. Incubation occurred for 5 minutes at room temperature. Cells were then washed twice in 15 ml of PPP, before being resuspended in just over 8 ml of PPP. Gamma activity was measured in the supernatant from the washes, and the total cells, using a dosimeter (Capintec radioisotope calibrator CRC-30BC, obtained from Delarese Electronics Ltd., Reading). Overall labelling efficiency was expressed as cell activity as a per centage of total activity. A 20μl aliquot was saved as a reference sample, and 2 ml of cell suspension was used per animal. Syringes and tubing were counted in the dosimeter after injection to correct for retained activity.

The isolation procedure used resulted in neutrophils with median purity of 95.1% (interquartile range 93.6 to 96.8) in a variety of experiments (n=40). Viability was > 99% as assessed by Trypan Blue exclusion. In the same collection of experiments, the median number of neutrophils injected per animal was 32.6 x 10^6 (interquartile range 25.7 to 43.0 x10^6). The labelling procedure had a median overall efficiency of 63.8 % (interquartile range 55.3 to 69.0).

2.2.4.1 Half-life and stability of In-wbc

The stability of binding was studied *in vitro* and *in vivo. In vitro,* ^{111}indium labelled neutrophils (In-wbc) were maintained in platelet poor plasma on a roller-mixer. An aliquot was removed at intervals and mixed with 2 ml of normal saline. This was centrifuged for 6 minutes at 300 g, and the supernatant (unbound) and the cell pellet (bound) fractions counted for activity in a gamma well counter.

From a linear regression fitted to the data points, 78.3 % of ^{111}In was cell-bound after 21 hours. This shows there was a slow release of ^{111}In from the cells *in vitro* over a time period similar to that used *in vivo.* However, the vast majority of the ^{111}In remained cell-bound, despite them being maintained in sub-optimal conditions.

For the *in* vivo determination, blood samples were obtained from the central ear artery directly into EDTA-containing tubes (Sherwood Medical, Crawley) 21 hours after the injection of In-wbc in 4 animals. These were centrifuged at 300g, and the supernatant (unbound) and the cell pellet (bound) fractions counted for activity in a gamma well counter. After 21 hours, 70.3 %, 87.9 %, 85.3 % and 82.0 % (mean 81.3%) of activity was bound, similar to that found *in vitro.*

The half-life of In-wbc was also measured *in vivo* in two cases. After intervals, blood samples were taken into weighed tubes containing EDTA, commencing at 1 hour. The bound and unbound ^{111}In fractions were counted as above, and from the weight of the sample bound ^{111}In per ml of blood were calculated. The half-life of disappearance was calculated by fitting an exponential curve.

$$y = b \cdot e^{-x.\,t}$$

where y = activity
t = time after injection of In-wbc
b = initial activity
x = constant

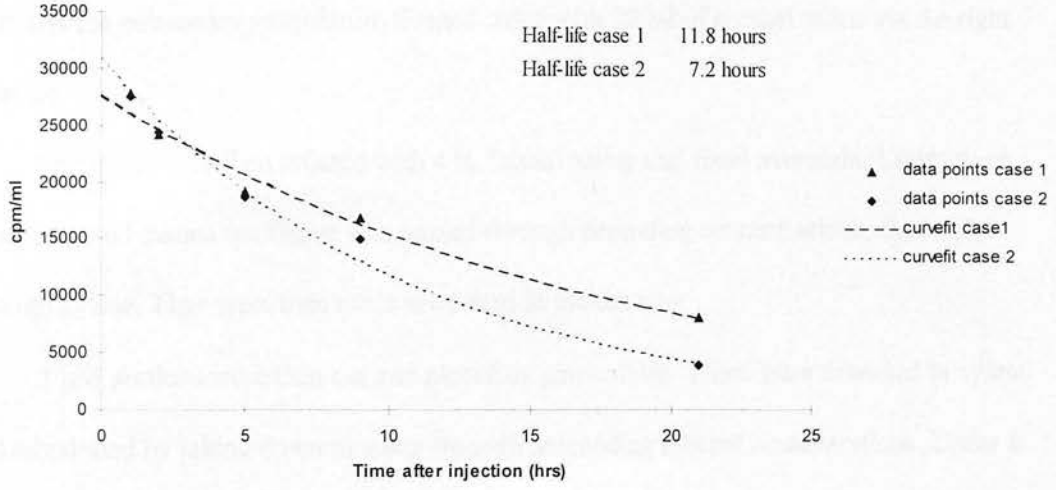
One half-life after injection, y = 0.5 b (by definition)

Therefore, the half-life, $t_{1/2}$, is given by the following formula;

$$t_{1/2} = \frac{\ln 2}{x}$$

Figure 2-2 *In vivo* circulating half life of ^{111}indium labelled neutrophils

The graph shows two experiments to determine the half-life of circulating In-wbc. Gamma well-determined cell bound ^{111}indium activity per gram of blood is shown, with a monoexponential curvefit in each case from which the half-life of circulating ^{111}indium labelled cells was determined.



No samples were taken in the first hour to allow equilibration of the injected In-wbc with the general blood pool. Using this method on two occasions, good exponential fits were obtained (F = 247.21 and 297.93, p = 0.001 and 0.001). Half-lives of 11.8 and 7.2 hours were obtained.

2.2.4.2 In vivo distribution of In-wbc

Autoradiographs of inflamed lung were performed to further confirm that ^{111}indium was predominantly cell bound in the lung after migration of labelled cells, as well as being cell bound in the circulation.

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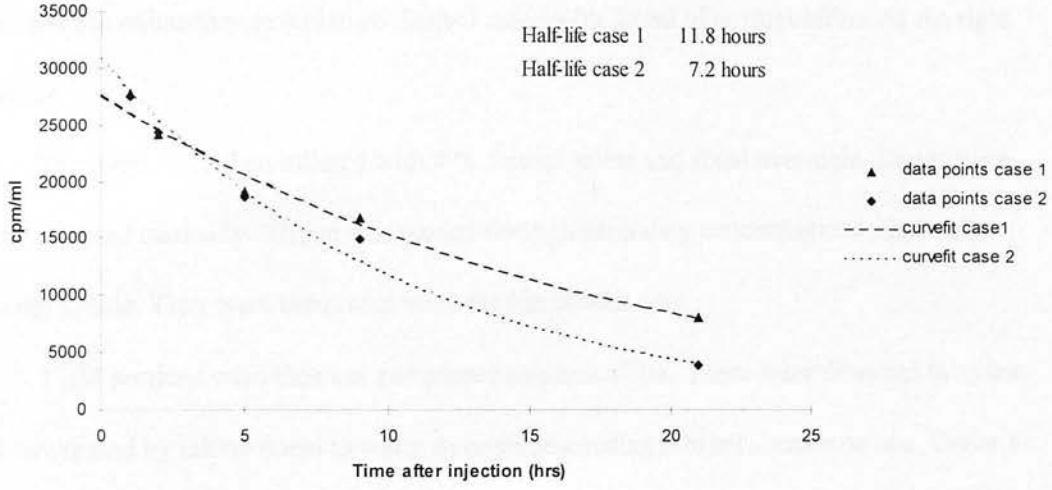

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6 hours after instillation of Strep. pneumoniae into the right cranial lobe (as described below), In-wbc were injected into a rabbit via the marginal ear vein. The animal was killed 21 hours later by overdose of intravenous pentobarbital. The heart and lungs were excised *en bloc,* and the pulmonary vasculature flushed twice with 30 ml of normal saline via the right ventricle.

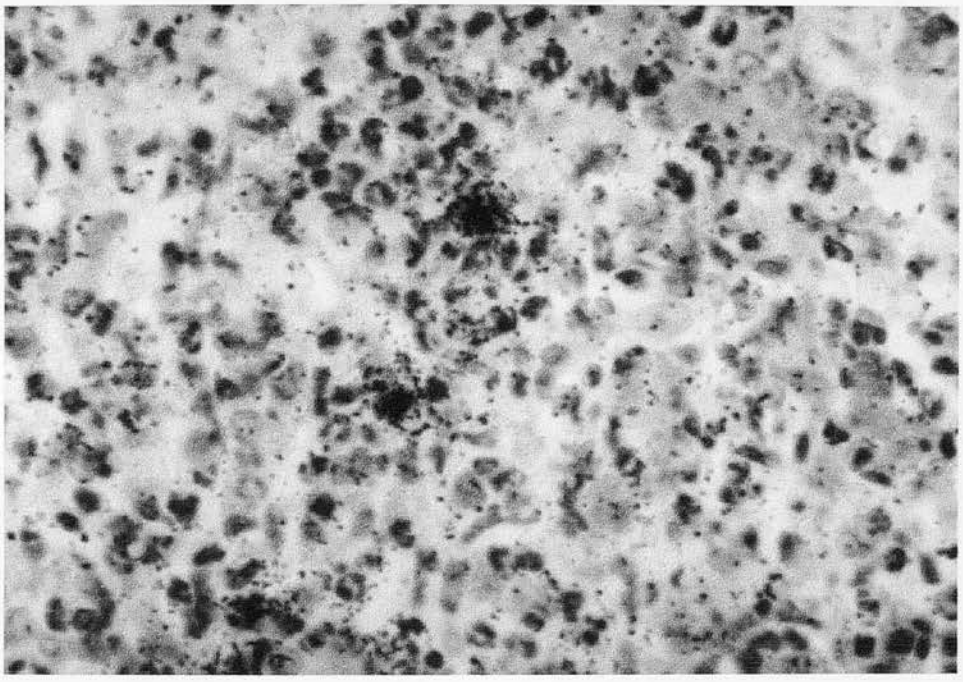
The lungs were then inflated with 4 % formal saline and fixed overnight. Lungs were then processed manually. Tissue was passed through ascending concentrations, then twice through xylene. They were then twice immersed in molten wax.

3 μM sections were then cut and placed on glass slides. These were dewaxed in xylene and rehydrated by taking down to water through descending ethanol concentrations. Under a safe light, Ilford K5 emulsion was melted in a Coplin jar in a water bath. Slides were dipped in this, and the reverse wiped and allowed to air dry. They were kept in a silica gel-containing light-tight box for 3 days, before developing for 7 minutes in 1 part Phenisol plus 4 parts distilled water at 18°c. After washing, they were fixed in 1 part Hypan fix in 4 parts of distilled water. Sections were then counterstained in haematoxylin and eosin (as below).

Examination of autoradiographic section showed ^{111}indium was predominantly cell associated. It was not always possible to determine the cell type that the radioactivity was associated with, because of density of silver grains. When the cell type could be determined, radioactivity was usually associated with neutrophils, although on occasion activity was associated with alveolar macrophages. This was presumably because of macrophage clearance of ageing neutrophils.

Figure 2-3 Autoradiographs of pneumonic lung

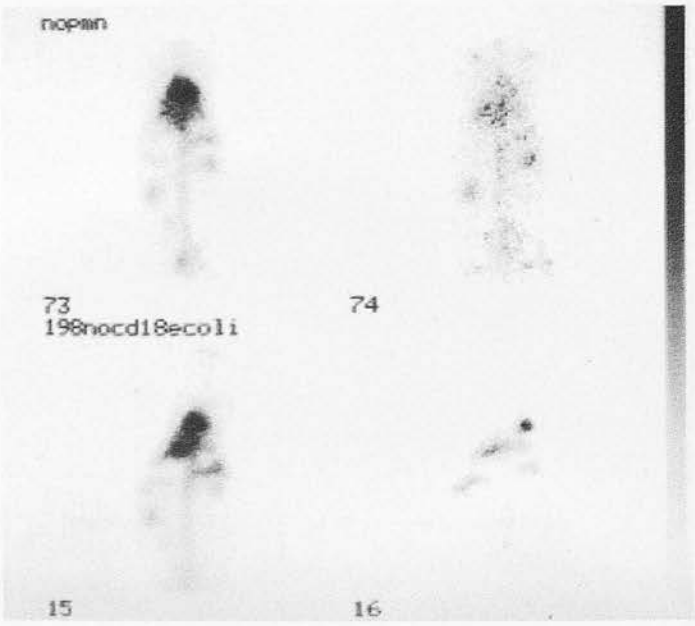
The figure shows autoradiographs of a rabbit lung following instillation of Strep. pneumoniae. After 6 hours, ^{111}indium-labelled neutrophils were injected. 21 hours later the animal was sacrificed and autoradiographs of the pneumonic lung performed.
Images show photomicrographs obtained at x 400 original magnification. Silver grains are seen to be predominantly localised to four cells, showing ^{111}indium remains mostly cell associated at the inflammatory site.



One animal had E. coli instilled (see below) into the right lung. After 6 hours, ^{111}indium chloride was chelated with troplonate as usual. However, instead of being incubated with neutrophils, this was injected directly into the subject animal. Autologous ^{99m}technetium-labelled erythrocytes (Tc-rbc) were injected as normal (see below). At the end of the study period, a gamma camera image was obtained as usual.

Figure 2-4 Gamma camera images showing ^{111}indium distribution with and without neutrophils

The figure shows four gamma camera images obtained from two animals. On the left are ^{99m}technetium gated images (images 15 and 73). On the right are simultaneously acquired ^{111}indium gated images (images 16 and 74). Images 15 and 16 are obtained from a rabbit with E. coli pneumonia, injected with In-wbc after 6 hours, and Tc-rbc 20 hours later, with images acquired after a further 5 minutes for equilibration. Images 16 and 74 are similar except that ^{111}indium chelated with tropolone was injected directly without incubation with neutrophils. Appearances are discussed in the text.



In figure 2.4, image 15 shows the ^{99m}Tc image from an animal with E. coli pneumonia, in the 6 hour study group. The heart, lungs, liver, spleen, both kidneys and great vessels can clearly be distinguished. This shows the ^{99m}Tc was distributed throughout the blood pool, as would be expected from its conjugation with erythrocytes. ^{111}In was distributed in a very different fashion. There was activity over the liver and spleen, but there was little activity over kidneys and great vessels. There was marked activity over the pneumonic lung field, but not over the rest of the lungs or the heart.

Image 73 shows the ^{99m}Tc image from the control animal injected with non-cell bound ^{111}indium. The Tc-rbc distribution in this animal was as for the animal injected with

^{111}indium pre-incubated with neutrophils. Panel 74 shows the activity in the ^{111}In image. This shows that when chelated ^{111}In-tropolonate was injected without incubation with neutrophils, it distributed in a very similar fashion to that of Tc-rbc. It distributes throughout the blood pool, with high activity throughout the lungs, heart, liver, spleen, kidneys and great vessels. No concentration of ^{111}In was seen over the inflamed lung.

This demonstrates that the distribution of the ^{111}In is dependent upon its binding to neutrophils. There is no concentration of free ^{111}In-tropolonate to inflamed lung on its own. Thus, any loss of ^{111}In from In-wbc will result in an under-estimate rather than an over-estimate of In-wbc recruitment.

2.2.5 ^{99m}Technetium labelling of erythrocytes

The procedure was based on that developed by Zimmer, 1977. This needed to be modified due to the different composition of the stannous kit used. For optimal labelling, 2-4 μg or less of stannous ion were required in 4ml of whole rabbit blood. Greater amounts gave declining efficiency. Thirty-two μg gave under 10% efficiency.

For the preparation of Tc-rbc, 4.5 ml of autologous blood was obtained from the central ear artery and collected into a 15 ml polyethylene centrifuge tube containing 1000 U of heparin (5000U/ml, Multiparin®, CS Pharmaceuticals, Wrexham). Ten ml of 0.9% sodium chloride for injections was added to a vial of Technescan® (containing 11.9 mg of sodium pyrophosphate and 3.4 mg tin(II) chloride, Mallinkrodt Medical UK, Round Spinney, Northampton) and 10 μl of this added to the whole blood. This was incubated for 10 minutes on a roller-mixer, then centrifuged at 300 g for 10 minutes. A 19 G hypodermic needle on a 2ml syringe (Plastipak®, Beckton Dickinson UK Ltd., Oxford) was used to aspirate 2 ml of packed cells. Two ml of sodium ^{99m}pertechnetate in normal saline (freshly generated from an Amertech II, 40 GBq generator, Amersham, Bucks., by Radiopharmacy, Royal Infirmary of Edinburgh), containing approximately 5 MBq of activity, was added to

the packed cells and mixed for a further 10 minutes on the roller mixer. Ten ml of normal saline were added and thoroughly mixed. This was centrifuged at 275 g for 6 minutes. Cells were washed a second time in normal saline before being resuspended in normal saline to 2 ml. The activity of the wash supernatants and the cells was assessed in the dosimeter for calculation of binding efficiency.

Using this procedure, a median of 86.1 % efficiency (interquartile range 73.8 % to 90.3 %) was obtained.

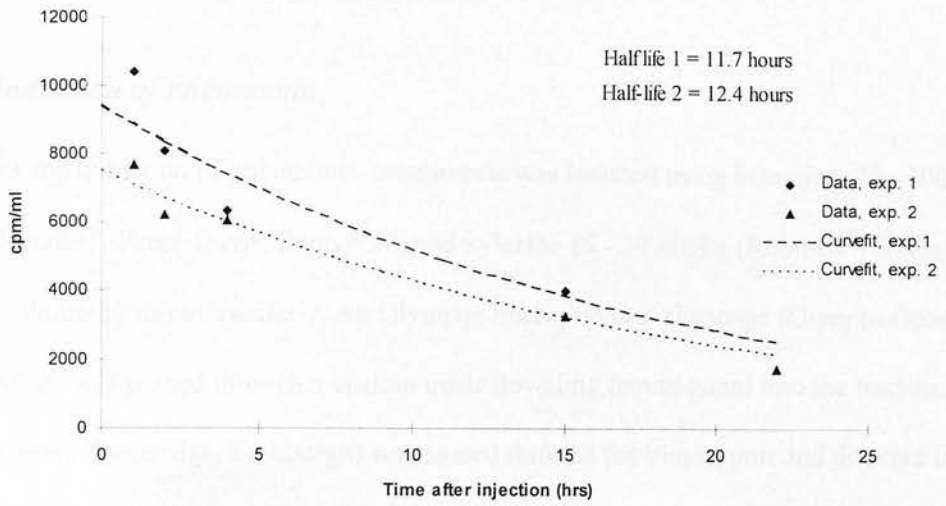
2.2.5.1 Half-life and stability of Tc-rbc

In vitro, Tc-rbc were maintained on a roller mixer at room temperature over 24 hours. Aliquots were removed at intervals, and centrifuged at 250g for 6 minutes. The bound (cell pellet) and unbound (supernatant) fractions were counted in a gamma well counter. Binding was >97% at all times.

In vivo, Tc-rbc were injected into 2 animals and approximately 1 ml of blood was taken from the central ear artery at intervals up to 22 hours into a weighed tube containing EDTA. Binding was measured as for the *in vitro* samples and was found to be over 97 % in all cases.

Figure 2-5 *In vivo* half life of ^{99m}technetium labelled erythrocytes

The graph shows cell associated ^{99m}technetium at intervals after the injection of autologous Tc-rbc in two animals. Monoexponential curves are fitted to the data, and half-lives calculated from these.



The calculated half-lives were only 11.7 and 12.4 hours. From this, the decline in activity during the 1hour duration of use in the experiments would be approximately 5.5 %. Extrapolating back to zero time gave initial apparent volumes of distribution of 131.6 and 165.4 ml.

2.2.5.2 In vivo distribution of Tc-rbc

As noted previously, Tc-rbc followed the expected blood pool pattern of distribution as determined by gamma camera images.

2.2.6 Preparation of bacteria

Bacterial plates were kindly prepared by Mr. Graham Williamson, Department of Medical Microbiology, Royal Infirmary of Edinburgh Trust. Streptococcus pneumoniae (NCTC 7465) was subcultured onto blood agar (Unipath Ltd., Basingstoke), and cultured overnight at 37^0 C in 5 % CO_2. Escherichia coli (NCTC 10418) was subcultured onto MacConkey's medium (Unipath Ltd., Basingstoke), and cultured overnight at 37^0 c in air.

Immediately prior to use, bacteria were suspended in PBS without cations, and the optical density of the solution adjusted to 0.25 at 550 nm using a spectrophotometer (Cecil CE292 series 2, Cambridge). Previous investigation by the Department of Microbiology shows this results in a suspension of 10^8 organisms per ml.

2.2.7 Induction of pneumonia

For the induction of pneumonia, anaesthesia was induced using ketamine, 70 - 100 mg/kg (Ketalar®, Parke-Davis, Pontypool) and xylazine 15 - 20 mg/kg (Rompun®, Bayer, Bury St Edmunds) intramuscularly. An Olympus fibreoptic bronchoscope (Olynpus Optical Co., London) was passed through a custom made doweling mouth guard into the trachea. A nylon catheter (Beveridge, Edinburgh) was passed through the biopsy port and directed into the most dorsal part of the right cranial lobe until it met resistance, then withdrawn slightly. 0.5 ml of bacterial suspension was injected through this, followed by 1.5 ml of air to clear the dead space. The passage of the bronchoscope and instillation of bacteria took about 30 seconds per animal, ensuring minimal stress.

Rabbits were nursed with the inoculated side dependent to help limit bacterial spread.

Using this protocol, rabbits remained systemically well, and continued to eat and drink normally. Usually, slightly diminished activity was seen only on the day following inoculation.

2.2.8 Overall pattern of experiment

Studies of neutrophil influx were carried out as shown in figure 2.6. After bronchoscopic instillation of either Strep. pneumoniae or E. coli, a period of time (x hours in figure 2.2) elapsed during which pneumonia proceeded without intervention. ^{111}indium-labelled neutrophils prepared as described were then injected via a 21 gauge Butterfly needle (Abbot Ireland, Ltd., Sligo, Ireland) into a marginal ear vein to test the migration of neutrophils from this time. Experimental groups were named by this time, the elapsed time

from induction of pneumonia to injection of In-wbc, although it should be realised that migration occurs in a time window commencing at this time. Unbronchoscoped animals were used as controls, and pneumonia was studied in 6, 30 and 80 hour groups.

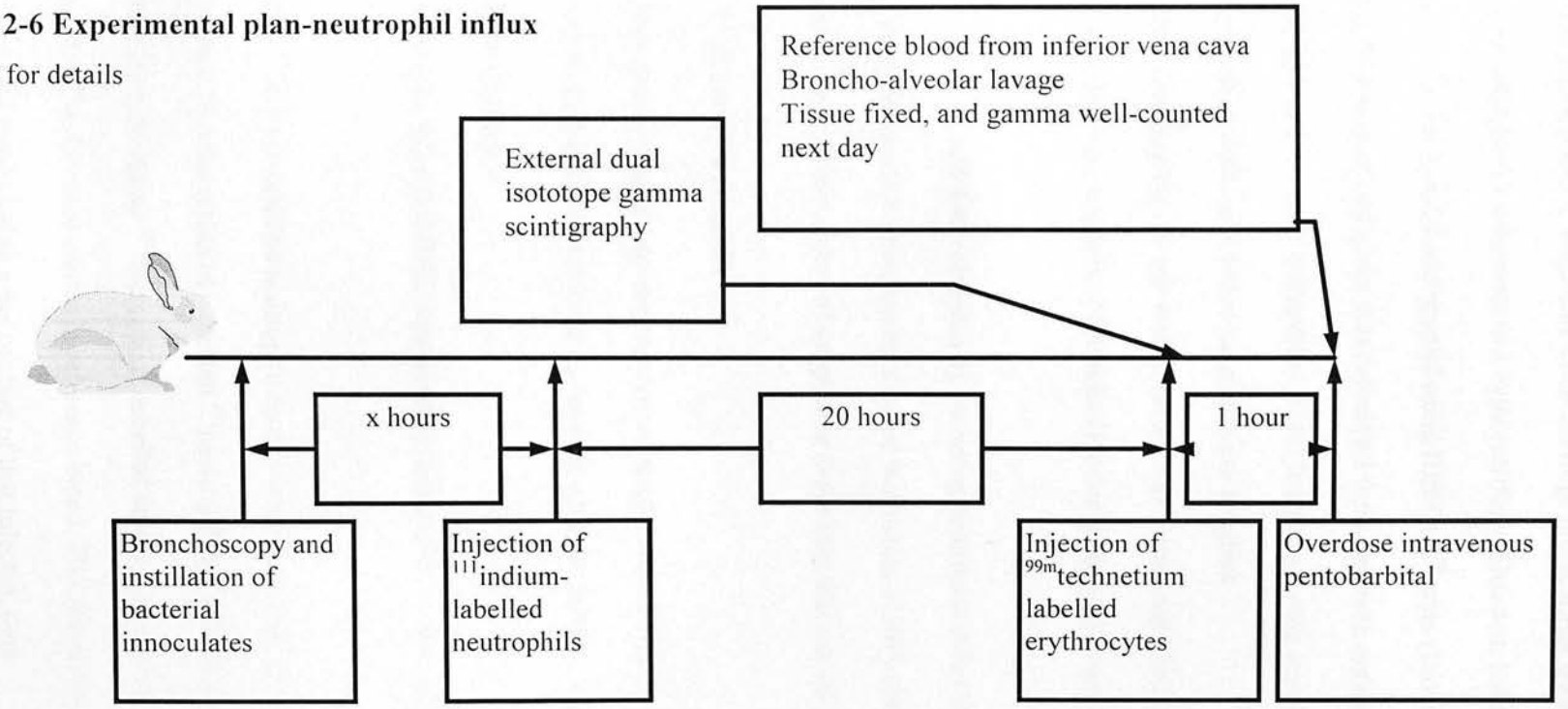
20 hours later, rabbits were re-anaesthetised using a similar protocol, and ^{99m}technetium-labelled erythrocytes prepared as described were injected, via a 19G butterfly needle, into a marginal ear vein. 5 minutes were allowed for equilibration before acquisition of a 300 second static dual isotope gamma camera image. 1 hour after the injection of ^{99m}techentium-labelled erythrocytes, animals were killed by slow intravenous injection of an overdose of Pentobarbital. The abdomen was then opened rapidly and a 1 ml reference blood sample was removed from the inferior vena cava into a weighed serum tube (Sherwood Medical, Crawley).

The abdominal vessels were sectioned and the heart and lung dissected free *en bloc.* 30 ml of normal saline were flushed through the pulmonary vasculature via a 19 G needle placed in the right ventricle, to rinse out free intravascular cells. The main bronchus was tied off using 2/0 Mersilk (Ethicon, Edinburgh). A portion of large bore extension tube was then secured in the trachea, and the right lung lavaged twice through this using 30 ml aliquots of chilled normal saline. Each aliquot was instilled and returned thrice to ensure good mixing, and kept on ice until processed.

The lungs were then fixed overnight by inflation with freshly prepared 4 % paraformaldehyde in PBS, without cations, to a pressure of 25 cm of water.

Figure 2-6 Experimental plan-neutrophil influx

See text for details



Broncho-alveolar lavage fluid was weighed to assess return. Of this, 5 ml was saved for gamma well counting. Duplicate cytocentrifuge preparations were made using 200 µl aliquots, at 300 rpm for 4 minutes in a cytocentrifuge (Shandon, Runcorn). They were fixed in methanol for one minute and stained using Diff-Quik® stain (Baxter Healthcare, Thetford, Norfolk). Differential cell count was calculated from the mean obtained from the assessment of at least 500 cells on each preparation. Total leukocytes were assessed by counting in a haemocytometer. Reference blood samples were weighed.

The following day, tissue was dissected into blocks and counted in a gamma well counter, together with aliquots of broncho-alveolar lavage, reference blood and reference In-wbc.

Controls values were obtained by assessing neutrophil influx into un-bronchoscoped rabbits, after preliminary experiments showed instillation of PBS alone produce inflammation one to two orders of magnitude lower than that caused by bacteria.

2.2.9 Expression of results

Bronchoalveolar lavage recovery of neutrophils and alveolar macrophages were expressed as total amount recovered on broncho-alveolar lavage, e. g.

Neutrophil recovery =

(Leukocytes per ml) x (ml BAL recovered) x (neutrophil fraction of total leukocytes)

The ^{111}indium-labelled neutrophil counts in a tissue were corrected for intravascular blood volume by subtraction of expected ^{111}neutrophil accumulation, which was in turn calculated from the tissue ^{99m}technetium-labelled erythrocyte counts and the ratio of ^{111}indium to ^{99m}technetium counts in reference blood. This corrected value was then normalised, and expressed as a per centage of that injected, thus:

Total injected activity = InTot

^{111}In activity in right lung = InRL

^{99m}Tc activity in right lung = TcRL

^{99m}Tc activity per gram reference blood = TcRef

^{111}In activity per gram reference blood = InRef

Tissue indium influx = {(InRL - (TcRL x [InRef / TcRef]) / InTot} x 100

Analogous calculations were performed using broncho-alveolar lavage ^{99m}Tc and ^{111}In activity to assess neutrophil influx into lavage, and using lung ROI ^{99m}Tc and ^{111}In activity with reference ROI ^{99m}Tc and ^{111}In activity to assess neutrophil influx by external scintigraphy.

Six hour values for total neutrophils and alveolar macrophages recovered by broncho-alveolar lavage, and neutrophil influx as assessed by tissue gamma well counting, external scintigraphy and broncho-alveolar lavage counting failed to differ significantly from normal distribution using the K-S test in either pneuS or pneuE. Parametric statistics were therefore utilised to assess differences in means, and an a priori decision was made to ustilise parametric testing in chapters 3 and 4, where methodolgy was very similar. In this chapter, differences in means were tested using one-way analysis of variance to test for an effect of time with Scheffe's *post hoc* analysis to test for significant differences from control (unbronchoscoped) values. Statistical values were calculated using the SPSS/PC+ 4.0.1 statistical package, with the exception of medians and interquartile ranges, which, being unavailable on this package, were calculated using Microsoft Excel version 5.0.

2.2.10 Histology

Ms. H. Caldwell, Department of Pathology, Edinburgh University Medical School, kindly prepared lungs for histological examination. After fixation over night in 4 % formal saline, the right cranial lobe was bisected along its longest axis, and each portion divided

into three. Remaining lobes were also divided into portions of roughly 1 cm^3. These were kept in formal saline for 2 to 3 weeks whilst radioactivity decayed. Lung pieces were transferred to Tissue-Tec cassettes and onto a Vacuum Impregnating Processing machine (VIP) overnight on a pre-programmed cycle, passing through various solutions of ethanol, xylene and wax. Tissue pieces were then removed and transferred to an ice tray and allowed to set into the wax.

Sections were then taken down to water, and stained in Harris's hamatoxylin for 3-4 minutes. After washing with water, sections were differentiated in 1 % acid alcohol for 5 - 10 seconds, and rehashed. They were then blued up in Scotts Tap Water for 1 to 2 minutes, and washed again, before staining with alcoholic/aqueous eosin, rewashed, dehydrated, cleared and mounted.

2.3 Discussion of methods

2.3.1 Pneumonia

Pneumonia was induced with either Streptococcus pneumoniae or Escherichia coli. These are typical examples of agents causing community acquired pneumonia (which is characteristically self-limiting) and nosocomial pneumonia (which is characteristically more damaging).

The method of instillation, using intrabronchial placement using fibre-optic bronchoscopy, was chosen for four reasons. Firstly, it mimicks aspiration, which is thought to be the usual mode of infection, much more closely than methods involoving aerosolisation of innoculates. Secondly, there is no surgical procedure involved, unlike techniques involoving tracheotomy, and it is thus less stressful. Thirdly, by causing a localised pneumonia, it allows a profound inflammation to be studied without an appreciable mortality; animals remain systemically quite well during the course of pneumonia. Fourthly, the position of the pneumonic area was constant. This may be important, as different areas

of the lung are characteristically affected by different disease processes. This is probably due to regional differences in ventilation and perfusion.

2.3.2 *Use of ^{111}indium-labelled neutrophils for studies of neutrophil influx, with particular reference to gamma camera imaging*

As has been alluded to previously, the number of tissue neutrophils present is a balance of their rate of influx and their survival and rate of clearance. Quantitation of neutrophil number by counting their number in a tissue or by measuring a marker of their presence such as total myeloperoxidase will thus only yield limited kinetic and mechanistic information. If a change in number cannot be attributed specifically to a perturbation of influx or clearance, then inferences drawn from studies of numbers alone may lead to dangerous errors.

A way to dissect influx more specifically is to introduce some labelled cells into a system. The behaviour of those cells at the time of introduction can then be studied by examining the distribution of the label. If this is done comparatively early after the introduction of the labelled cells then this will be predominantly an influx signal. Even if there is some breakdown of the labelled cells in situ, or they begin to be cleared (for instance by macrophages) at an inflammatory site, this may permit their continued detection, presuming there is no significant transfer of free label or scavengers of labelled cells away from the inflammatory site during the course of study.

In the past, a variety of labels have been used. However, ^{111}In labelled neutrophils (In-wbc) have been widely used both in clinical and research applications. (Peters et al, 1983, Peters, 1992, MacNee et al, 1989, Rinaldo et al, 1988 and Haslett, et al, 1987). The isotope ^{111}In is an attractive cell labelling agent as several techniques exist which permit its use for labelling leukocytes with high efficiency. It emits 173 and 247 keV gamma rays with high external photon yields, which is suitable for both gamma well counting and

external scintigraphy. It has a half-life of 67 hours which is long enough to track cells, but is short enough in clinical studies to prevent excessive dose exposure to subjects (McAfee et al, 1984, and Thakur et al 1984). Generally, labelling is found to be stable and specific. The main concern with studies using labelled cells is that the label perturbs the cells in some way, and this modifies their *in vivo* behaviour. In addition, the labelling procedures used to date are not specific for neutrophils. Whilst for clinical studies the use of mixed cell populations may suffice, particularly if the patient has a leukocytosis (Palestro et al, 1992), this non-specificity means neutrophils must first be isolated from whole blood. This in turn may induce subtle perturbations that could affect *in vivo* behaviour.

2.3.2.1 Neutrophil isolation.

The effect of different neutrophil isolation procedures has been studied in detail (Haslett et al 1985). Using human blood these workers found that neutrophils prepared by erythrocyte sedimentation in dextran followed by separation on a discontinuous plasma-Percoll density gradient produced cells that were minimally primed or activated. This was assessed by the minimal shape change produced and the minimal priming exhibited for the production of superoxide or lysosomal enzyme release, as well as the maximal response to chemotactic agents. Preparation by other methods, and in particular separation on Ficoll-Hypaque gradients was found to produce cells which were primed or partially activated, behaving as if exposed to a low dose of bacterial lipopolysaccharide.

Later, this method was adapted to the isolation of rabbit cells (Haslett et al, 1987). (The rabbit equivalent of neutrophils are actually heterophilically staining, but are referred to here as neutrophils in analogy with, and for easy comparison to other species). The rabbit neutrophils are of slightly different density from human neutrophils, necessitating slightly different density gradients. In addition, their density overlaps with that of erythrocytes. This

necessitates the use of a hypotonic lysis stage after removal of the purified neutrophil population from the density gradient.

Although Lane et al, 1982, found that hypotonic lysis caused rabbit neutrophils to circulate with a different pattern from cells that had not undergone this procedure, Haslett et al, 1987, found neutrophils underwent identical patterns of circulation with or without a lysis step. Unlike Haslett's group, Lane's group used Ficoll-Hypaque for cell separation this may have primed or partially activated the cells. This may also have rendered them more susceptible to damage by hypotonic lysis. Additionally, they used oxine rather than tropolonate for indium chelation, and this may be a further, synergistic mode of injury (see below).

For studies described in this thesis, cells were therefore isolated using the method of Haslett et al.

2.3.2.2 Labelling method

For labelling purposes, indium is first chelated with a lipophilic compound. This allows indium free access to the cell. There, the indium separates from the chelating agent and binds to proteins in the cytosol or in the nucleus (Thakur, 1977). It is a worry that the nuclear binding in particular could cause radiotoxicity to the neutrophil. In fact, the neutrophil has turned out to be remarkably resistant to radiation effects (Holley et al, 1974). High enough doses do prove to produce morphological changes and to inhibit chemotaxis and phagocytosis. The doses required to produce any effect are very much higher than those to which cells are typically exposed in labelling studies (Segal et al, 1978).

Two main chelators of indium have been used; oxine (8-hydroxyquinoline) and tropolone (2-hydroxy-2,4,6-cyloheptantrien-1-one). Both of these are known to have some toxicity. Oxine has been used as a topical fungicide, antiseptic, antiperspirant and spermicide, whilst natural copper chelates of tropolone give antibacterial properties to cedar

and redwood (McAfee et al, 1984). With these facts in mind there is clearly cause for concern that these compounds are toxic when used for labelling. In addition, indium-oxine labelling in the presence of plasma gives very high indium-transferrin binding (Thakur et al, 1977). This means cell labelling must be done in plasma-free conditions. Removing cells from their natural environment may also cause some abnormality of function.

To assess the toxicity of these compounds as part of the cell labelling process, both *in vitro* and *in vivo* studies have been performed.

2.3.2.2.1 *In vitro* studies

Neutrophil labelling using ^{111}indium-oxine must been done in plasma free conditions because of the marked ability of the chelate to label transferrin. This has given concern that this isolation from its normal environment may predispose the neutrophil to damage during the process. Although ^{111}indium-tropolonate shows some affinity for transferrin too, this is sufficiently low for efficient cell labelling in the presence of plasma when cell concentrations are high (Danpure et al, 1982, McAfee et al, 1977).

When examined *in vitro*, there have been contradictory data on the toxicity of the two compounds. For example, chemotaxis and phagocytosis were found to be impaired by oxine but not tropolonate in one study, (Burke et al, 1982), whereas another study found there to be no difference in chemotaxis *in vitro* or *in vivo,* with excellent viability as assessed by ability to exclude vital dyes (Das et al, 1988). Others have found ^{111}indium-oxine-labelled neutrophils to show normal chemotaxis and phagocytosis, with low basal and high stimulated ability to generate superoxide (Selby et al, 1991).

The apparent contradiction is probably explained by subtle differences in techniques and reagents. Burke, et al, noted that cadmium produced toxic effects on the neutrophil in tiny doses. It may be present together with other trace elements as part of the ^{111}indium preparation, as it forms part of the target in its generation.

Although no clear differences in toxicity have been demonstrate, the fact that labelling can take place in more physiological conditions (i.e. the presence of plasma) using tropolonate means it is nevertheless preferred on theoretical grounds.

2.3.2.2.2 *In vivo* studies

Damaged neutrophils are likely to be removed early from the circulation. Thakur et al, 1977, confirmed this by showing heat damaged In-wbc were removed from the circulation on passage through the lungs. The circulating half-life has therefore been used as an index of neutrophil health.

Cohen et al, 1982, found *ex vivo* ^{111}indium-oxine-labelled neutrophils and *in vivo* tritium pulse labelled cells had very similar half-lives of 4.08 and 4.03 hours respectively. Other studies have found values of 5.6 $\pm$ 2 hours using ^{111}indium/oxine labelling (Lane et al, 1982), 4 hours using ^{111}indium/tropolone labelling (Haslett et al, 1987), and 6.7 hours using ^{51}chromium labelling (Doerschuk et al, 1987). The overall consistency of values despite the use of varying techniques suggests the cells circulate normally and are thus minimally damaged by labelling.

Ex vivo exposure of ^{111}indium-labelled neutrophils to trace amounts of LPS produces increased pulmonary retention of these cells on reinjection (Haslett et al, 1987). If labelled cells were significantly damaged, such subtle effects would be masked.

Reinjected In-wbc are often 'held up' in the lung (e.g Thakur et al, 1977, MacNee et al, 1989, and Warshawski et al, 1986). Some have argued pulmonary hold up of In-wbc is wholly artefactual. In a large series of clinical studies, various degrees of lung hold up and subsequent hepatic and splenic uptake could be related to the technique of labelling, with minimal hold-up seen with ^{111}indium/tropolone labelling, which was therefore felt to be the optimum agent (Saverymuttu et al, 1983).

Hold up might also be caused by clumping of labelled cells prior to reinjection, particularly as autoradiography has demonstrated clumped cells are more likely to be heavily labelled (Puncher and Blower, 1995), but autoradiography of the lung did not demonstrate this (Lichter et al, 1984).

Nevertheless, studies examining changes in initial holdup have produced coherent data. Initial holdup is increased, and subsequent washout decreased, after cigarette smoking (MacNee et al, 1989) and during ARDS (Warshawski et al, 1986). This data is consistent with the proposed involvement of the neutrophil in smoking-induced emphysema and in ARDS.

Neutrophils are undoubtedly physiologically delayed during passage through the lung, as has been demonstrated by studies utilising non-labelled as well as labelled cells (see general introduction). Release of sequestered In-wbc from the lungs occurs after adrenaline infusion (Haslett et al, 1987, and Muir et al, 1984), mirroring normal physiology.

There is also an increased pulmonary In-wbc sequestration after inflammatory stimuli, mirroring the behaviour of unlabelled cells. Injection of pneumococcal lysates produced In-wbc sequestration specifically in the lungs, but not in the brain, heart, liver, spleen or kidneys (Goldblum and Reed, 1985). This selectivity is not merely the result of retention at the first vascular bed that is encountered; whilst intra-arterial injection of In-wbc does result in transient local neutrophil retention, there is also pulmonary retention, and the rate of washout from the lungs is much less than from the local vascular bed (Williams et al, 1989). Likewise, during dialysis, In-wbc are selectively sequestered in the lung (Becker et al, 1989).

Thus although excessive pulmonary sequestration may reflect neutrophil damage, In-wbc also sequester in a way that mirrors normal cells.

There is no doubt that neutrophils are recruited to a wide variety of inflammatory foci. It has been suggested that on the basis of clinical observation that In-wbc prepared using

tropolone chelation may localise more rapidly than those using oxine chelation (Peters et al, 1983), no trial has ever shown a difference between the two.

Overall, *in vivo* studies show that In-wbc neutrophil using either technique of chelation circulate with normal half-lives, sequester reversibly in response to appropriate stimuli and localise to sites of inflammation. Detailed studies of pulmonary kinetics suggest a marginal benefit to using tropolone as chelating agent.

2.3.2.3 Cell labelling and behaviour in this study

Tropolonate was used as the chelating agent in the current study. With this agent, good labelling efficiencies were obtained, with excellent cell viability. The calculated half-lives of 11.8 and 7.2 hours are relatively long but of similar order to those found by others. As discussed, cell damage during labelling is likely to lead to abbreviation of half-life rather than prolongation, suggesting cells used in these experiments were undamaged.

^{111}indium-labelled neutrophils used in this study did show some loss of activity during the course of the experiment, around 20 % being lost *in vivo* and *in vitro.* This is higher than that reported by others, e.g. Haslett et al,1987, found more than 98 % remained cell bound after 24 hours using the same methods. The reason for the discrepancy is not clear.

Importantly, chelated ^{111}indium did not localise to actively inflamed areas if not previously incubated with cells at high concentration. There was no loss apparent from urine or faeces when cages were checked with a scintillation counter. It seems likely that the ^{111}indium-tropolone binds to transferrin and remains intravascular. Autoradiography confirmed that radioactivity within the lung was largely cell-associated. The net result of this will be to underestimate the recruitment of neutrophils slightly (each neutrophil will carrying less activity), but there is no reason to suppose any treatment group will be differentially affected. Thus comparisons should still be valid.

The use of neutrophils from donors could potentially give erroneous results if rabbits were sensitised to histocompatability mismatches. Such effects have been described in humans, where early lung retention was increased in alloimmunized patients (Dutcher et al, 1990). There is no reason to think the rabbits in this study would be alloimmunised, not previously having received transfusion of blood products, and heterologous neutrophils were found to behave indistinguishably from autologous cells in other studies in the rabbit (Haslett et al, 1987).

2.3.2.4 ^{99m}technetium-labelled erythrocytes

In many vascular beds, inflammation is associated with vasodilation. This results from prostaglandins, calcitonin gene-related peptide (CGRP) and other substances released locally (Brain et al, 1985) as well as by neural reflexes. It allows defensive macromolecules and cells increased access to the inflammatory site. In the lung, the blood flow is often found to decrease (Downey et al, 1988). This may help protect against the hypoxia that would be the result of perfusion of unventilated lung units. These vascular flow changes might well be associated with changes in intravascular volume. In order to correct for this, Tc-rbc were infused. This allowed calculation of the expected neutrophil blood pool at the pneumonic site, and subtraction of this from the neutrophil total to give a measure of specific recruitment.

Tc-rbc prepared using a minor modification of the method of a clinically utilised *in vitro* method (Zimmer, A. M., 1977) showed excellent stability of binding. However, the *in vitro* half-life of the erythrocytes was very short, determined to be around 12 hours on the two occasions studied. This no doubt indicates a degree of *in vitro* damage during preparation. However, this represents only just over a 5 % loss during the 1 hour between injection and euthanasia. Effete erythrocytes are cleared by the spleen, and counts of activity in this organ were not used in any calculations. There is no reason to presume the other

erythrocytes were not evenly distributed around the circulation. This is supported by the initial apparent volume of distribution which was compatible with the intravascular volume. If damaged erythrocytes were immediately sequestered in the lung, the apparent volume of distribution would be large.

2.3.2.5 Pattern of neutrophil recruitment, and location of neutrophil excess

The distribution of In-wbc was assessed 21 hours after injection. The methods used measured the neutrophils accumulating in the right, pneumonic lung in excess of that expected from its blood content, compared to neutrophil content of peripheral blood. This therefore potentially includes sequestered neutrophils, interstitial neutrophils and airspace neutrophils. The relatively long interval chosen helps to ensure the signal was predominantly related to migration rather than sequestration of neutrophils. The interval between injection of In-wbc and assessment of their distribution is at least 2 circulating neutrophil half-lives. The sequestered pool is in dynamic equilibrium with the circulating pool, so the sequestered pool should also be small at the end of this time.

For similar reasons, the majority of recruitment of labelled neutrophils is likely to commence early in this time period, for the simple reason that there will be far fewer available for recruitment towards the end of the study period. Thus although recruitment is assessed over a 21 hour time window, measures will be heavily weighted towards recruitment occurring early in this time frame.

Some local neutrophils may reach the airspaces not by active recruitment, but by haemorrhage. To a large extent this will not be corrected by the use of Tc-rbc to assess blood pool, as these are only injected in the last hour of the study. However, although histological sections sometimes show some evidence of intra-alveolar haemorrhage, the number of neutrophils was always greatly in excess of the number of erythrocytes in the

inflamed lung. As the converse is true in the blood, the vast majority of neutrophils must be actively recruited and thus any such error will be negligible.

2.4 Results

2.4.1 Macroscopic and histological appearance of pneumonia

Macroscopically, both pneuS and pneuE produced a consistent pattern of pneumonia. A darkened, indurated area of inflammation was produced about 1 cm in diameter in the right cranial lobe. The area of induration sometimes extended into the apex of the caudal lobe on the right, and sometimes encroached slightly into the intermediate lobe. There was never any gross inflammation in the left lung.

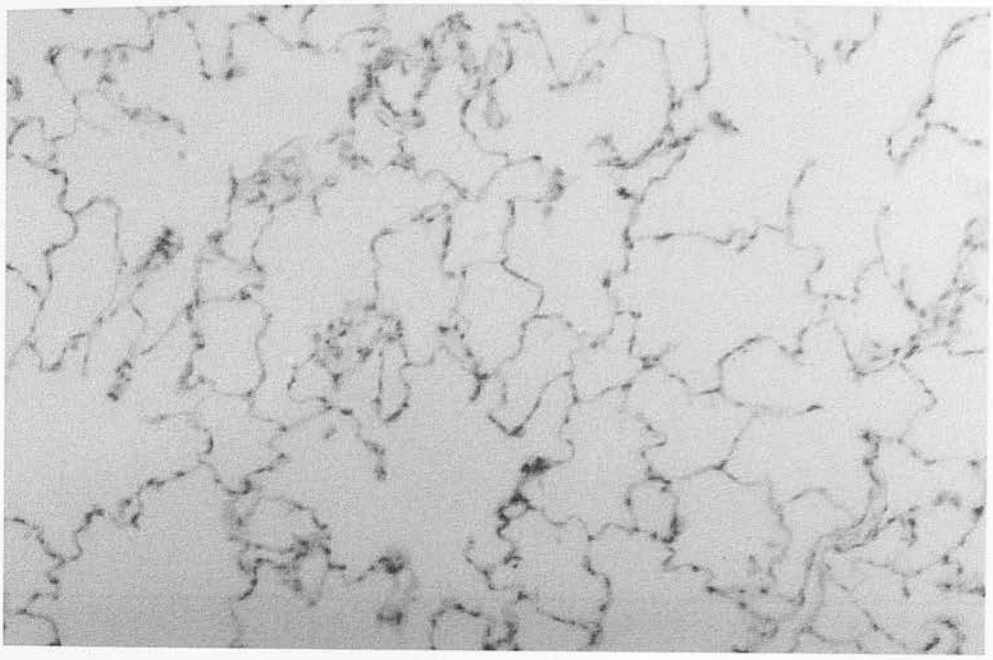
Histologically, pneuS and pneuE produced broadly similar patterns of pneumonia. No clear difference was observed between inflammatory cell numbers in the two types of pneumonia, though the localised nature of the lesion, and the variation of histological appearances across the area of inflammation, would necessitate the use of extensive morphometry to convincingly demonstrate other than gross differences in cell numbers. Normal rabbit lung showed fine alveolar walls with a paucity of intra-alveolar cells (figure 2.7). In sections taken from areas of from the 6 hour group (therefore killed at 27 hours after induction of pneumonia), there was a dense cellular infiltrate into the airspaces so that in large areas alveoli were entirely cellular and contained no air (figure 2.8). At this time, large numbers of neutrophils could be seen, although there appeared to be a considerable increase in mononuclear cells too. It appeared there were areas in which the neutrophil infiltrate would predominate, and others in which the mononuclear cells would predominate. The alveolar walls were clearly more cellular too. They contained mostly mononuclear cells representing macrophages, alveolar epithelial cells and fibroblasts, but some neutrophils were present too.

In the 30 hour group (killed at 51 hours), there appeared to be proportionately fewer neutrophils in the infiltrate (figure 2.9), whilst in the 80 hour group (killed at 101 hours) there were comparatively few neutrophils left (figure 2.10). A dense mononuclear cell infiltrate remained. In a few animals examined at after 189 hours, the inflammatory infiltrate was composed almost entirely of mononuclear cells (figure 2.11). It was becoming much less marked, and overall there was the impression that the lung was more aerated, though formal morphometric analysis was not undertaken.

The appearances thus are seen to be those expected during an acute pneumonia. There is an initial, profound inflammatory state characterised by significant numbers of neutrophils. This is followed by a predominantly mononuclear infiltrate, and then resolution commences.

Figure 2-7 Histology of normal lung

The photomicrographs show the appearance of normal lung. The upper photomicrograph is at an original magnification (om) of x 100, the lower plate at an om of x 400. The alveolar walls are thin and there is a paucity of intra-alveolar cells.



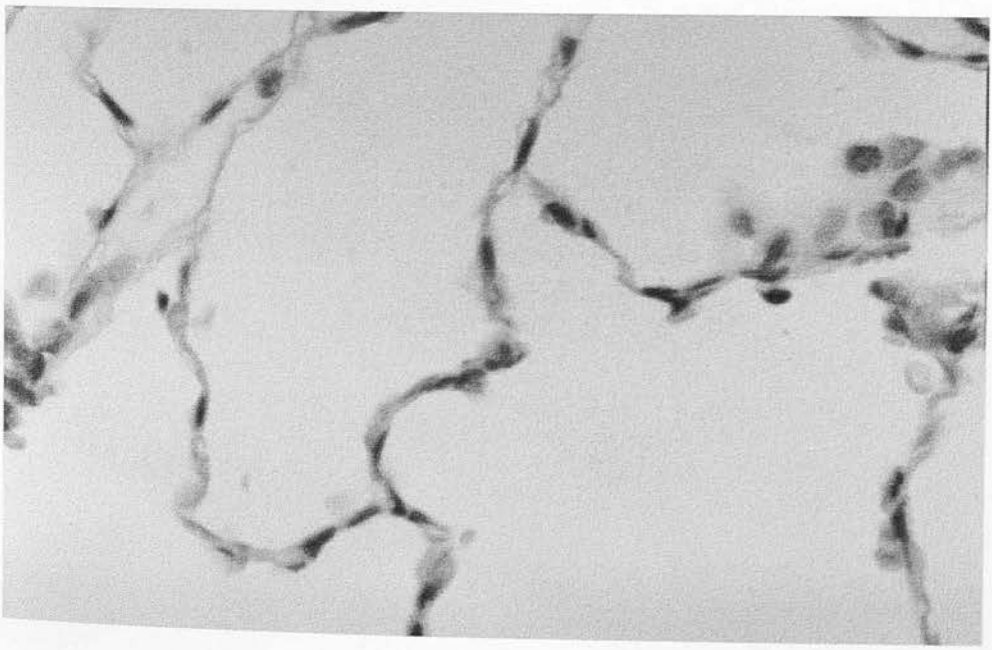
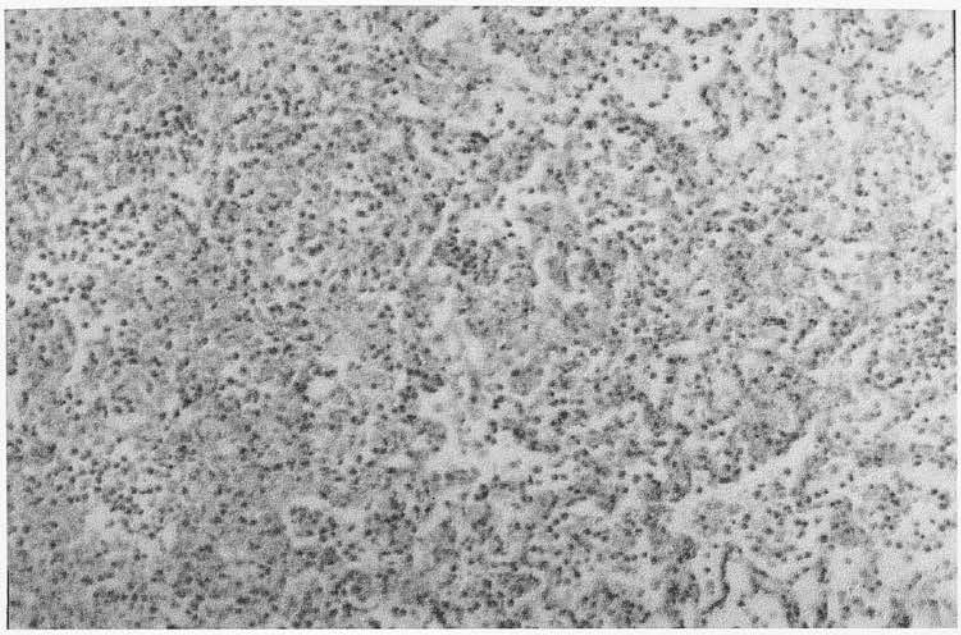


Figure 2-8 Histology of pneumonia after 27 hours

The photomicrographs shows the appearance of pneuS after 27 hours. There is an intense inflammatory infiltrate, with a marked predominance of neutrophils. (Upper om x100, lower om x400)



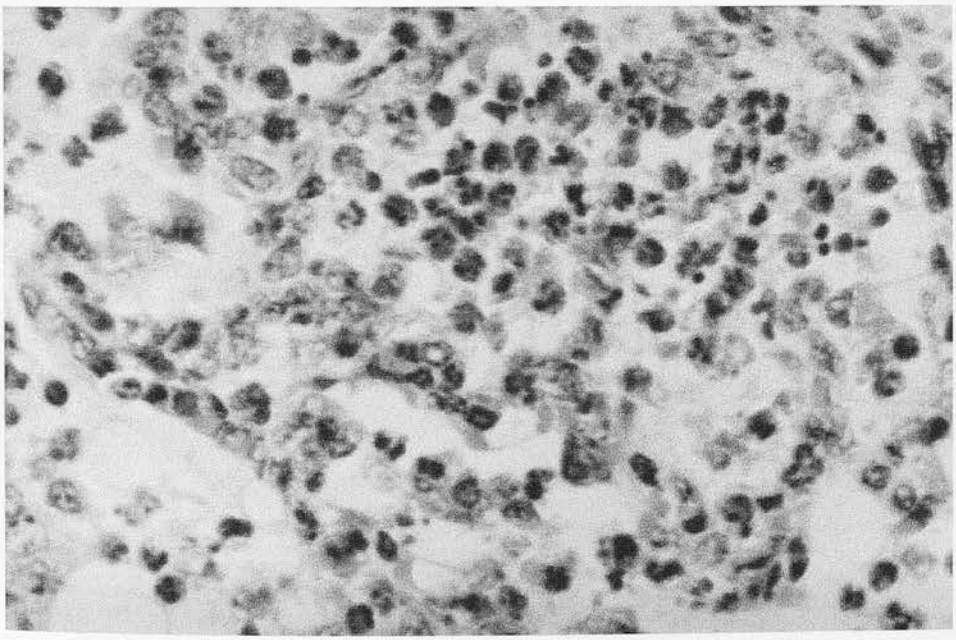


Figure 2-9 Histology of pneumonia after 51 hours

The photomicrograph shows the appearance of pneuS after 51 hours. There is an intense inflammatory infiltrate, with neutrophils less prominent than formerly. (Upper om x100, lower om x400).

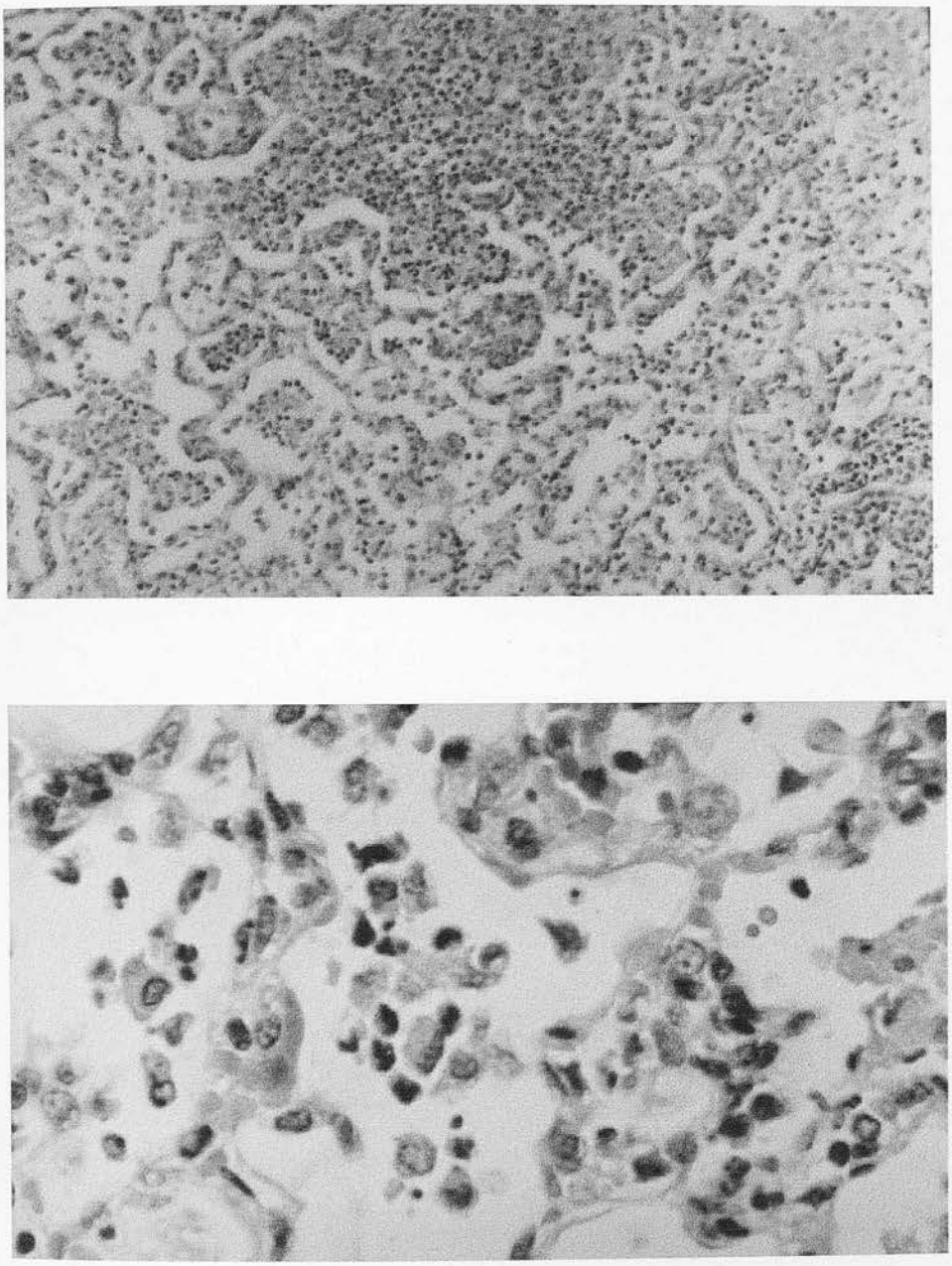


Figure 2-10 Histology of pneumonia after 101 hours

The photomicrograph shows the appearance of pneuS after 101 hours. The inflammatory infiltrate is less marked, and neutrophils are comparatively scanty. (Upper om x100, lower om x400).

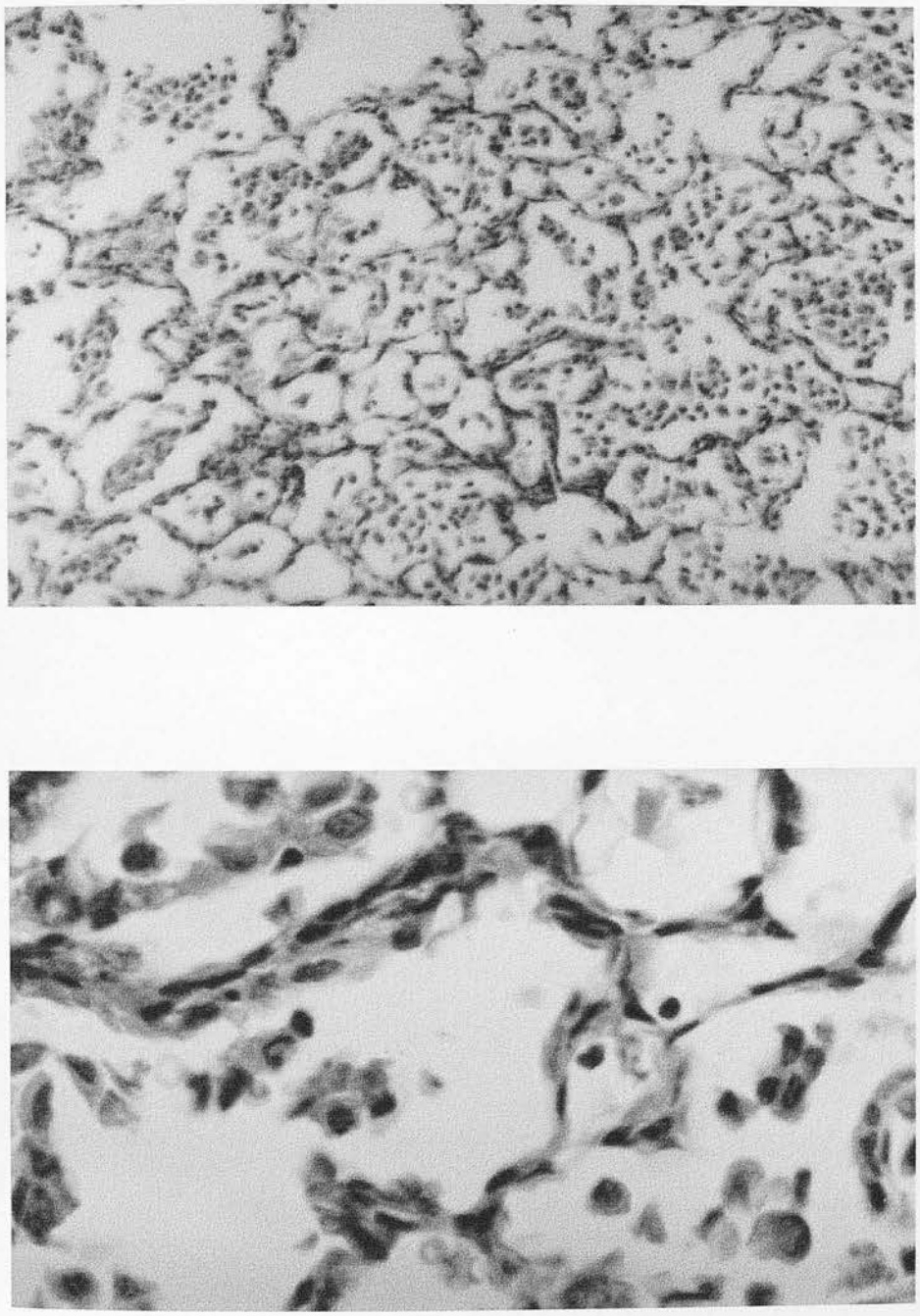
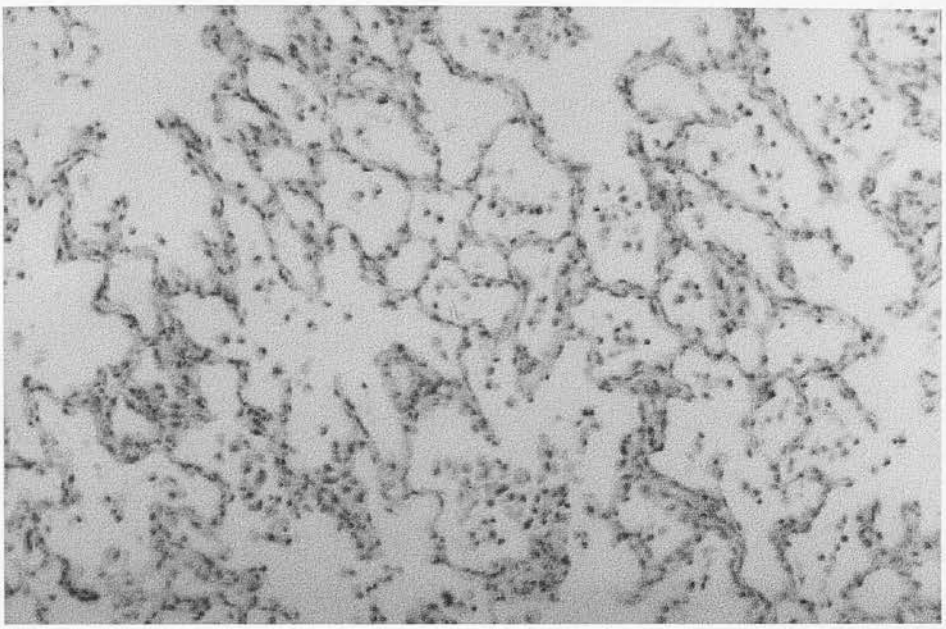
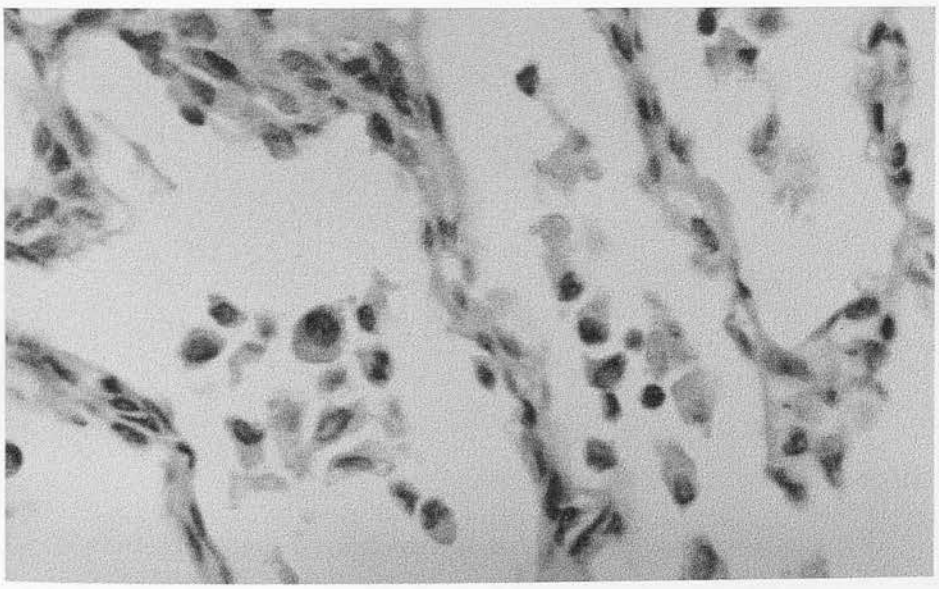


Figure 2-11 Histology of pneumonia after 189 hours

The photomicrograph shows the appearance of pneuS after 189 hours. The inflammatory infiltrate has clearly declined, and the majority of inflammatory cells are mononuclear cells. (Upper om x100, lower om x400)

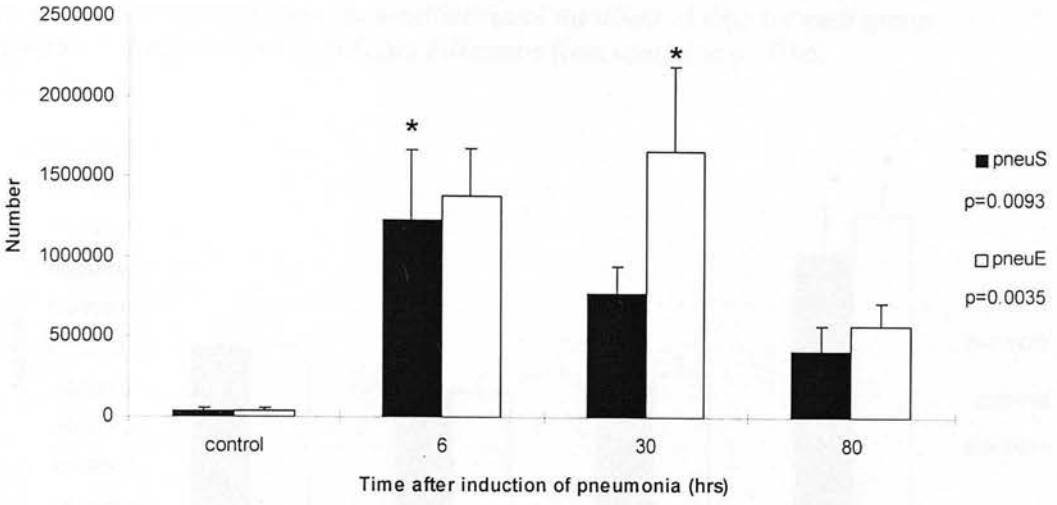




2.4.2 BAL neutrophil content

Figure 2-12 Broncho-alveolar lavage neutrophil recovery

The graph shows the total number of neutrophils recovered from BAL at various time points in each type of pneumonia. The graphs show means, error bars SEM, n=14 to 17 per group. The legend shows the significance of the effect of time for each group (ANOVA). * represents a significant difference from control at p <0.05.



In control rabbits that were unbronchoscoped, there were very few neutrophils in the bronchoalveolar lavage (mean 0.42 x 10^5 ± 0.11 x 10^5 SEM). In PneuS, ANOVA showed that there was a significant effect of time (p=0.0093). The maximum number of neutrophils in BAL were found in the 6 hour group, i.e. 27 hours after pneumonia induction (12.38 x 10^5 ± 4.36 x 10^5). *Post hoc* analysis using Scheffe's test indicated this to be significantly raised above control levels. At later time points, the BAL neutrophil levels were not significantly elevated above controls. Overall, the trend was for a peak at the earliest time point, followed by a decline.

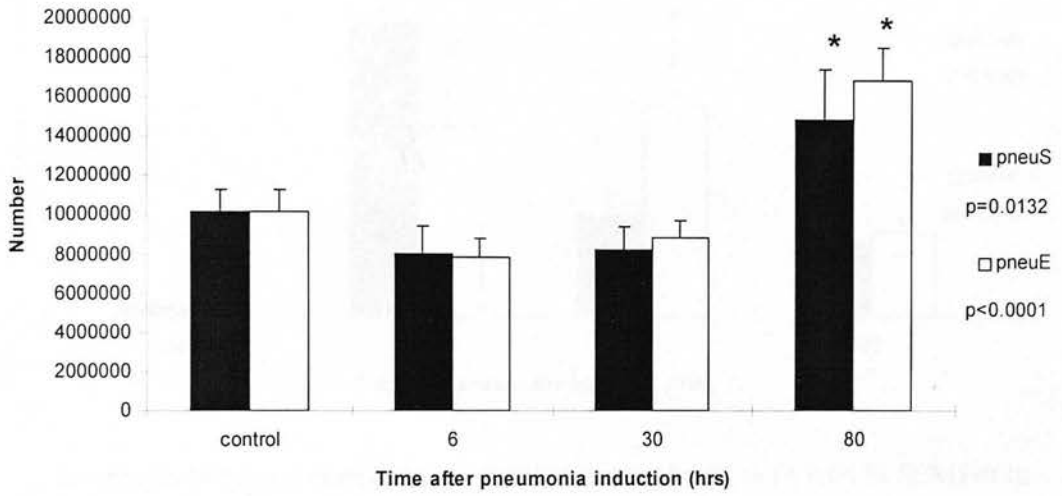
In pneuE, there was also a significant effect of time on neutrophil number in BAL (p=0.006). The highest number was found in BAL taken from the 30 hour group, i.e. 51 hours after the onset of pneumonia (16.74 x 10^5 ± 5.27 x 10^5). *Post hoc* analysis showed that neutrophil numbers in BAL were significantly elevated above control in the 6 and 30

hour groups. Overall, the trend was for an increase that was sustained so it was at least as high in the 30 hour group as the 6 hour group before declining.

2.4.3 BAL alveolar macrophage content

Figure 2-13 Broncho-alveolar lavage macrophage recovery

The graph shows the total number of alveolar macrophages recovered from BAL at various time points in each type of pneumonia. The graphs show means, error bars SEM, n=14 to 17 per group. The legend shows the significance of the effect of time for each group (ANOVA). * represents a significant difference from control at $p < 0.05$.

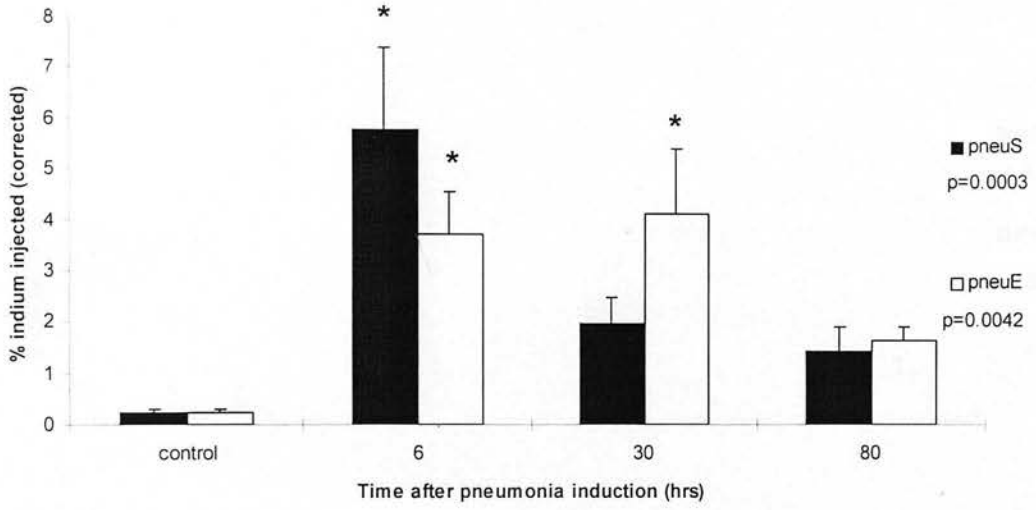


Large numbers of alveolar macrophages were recovered from BAL of control animals ($10.22 \times 10^6 \pm 1.09 \times 10^6$). There was a significant effect of time in both pneuS and pneuE ($p=0.0132$ and $p<0.0001$ respectively). In both types of pneumonia, there was little change until the 80 hour group, i.e. 101 hours after the induction of pneumonia. In this group there was a significant increase in the number of BAL alveolar macrophages in both pneuS and pneuE.

2.4.4 Tissue In-wbc influx

Figure 2-14 Neutrophil influx assessed by gamma well counts

The graph shows the amount of neutrophil influx into the right, pneumonic lung at different time points in each type of pneumonia, as assessed post mortem by tissue well counts. The graphs show means, error bars SEM, n=14 to 17 per group. The legend shows the significance of the effect of time for each group (ANOVA). * represents a significant difference from control at $p < 0.05$.

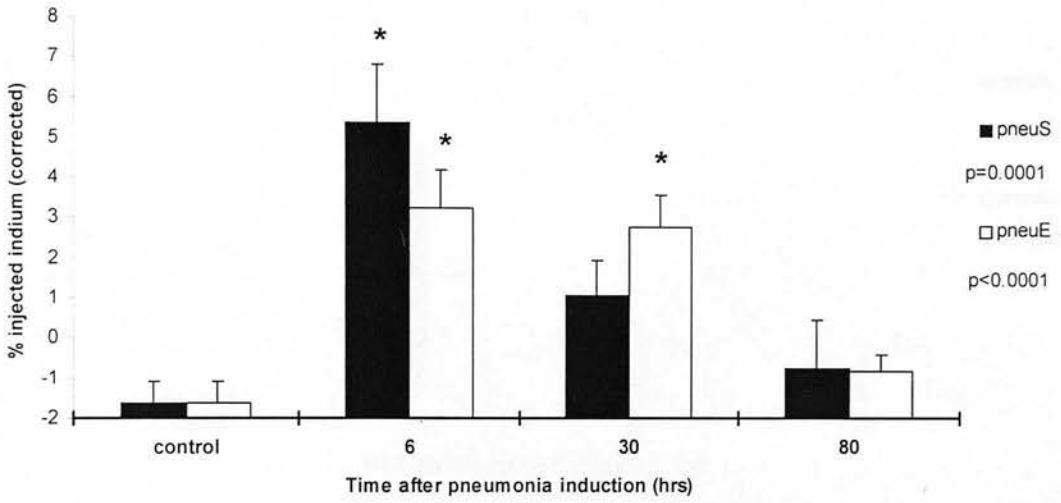


In unbronchoscoped control animals, only a mean of 0.25 % (± 0.04 % SEM) of In-wbc were recruited to the lung. In pneuS and pneuE there was a significant effect of time ($p = 0.0001$ and $p < 0.0001$). In pneuS there was a significant increase in the 6 hour group (mean 5.78 % ± 1.62 % SEM). Following this there was a marked decline. In pneuE, there was a significant increase not only in the 6 hour group but also in the 30 hour group. In fact, the mean influx of In-wbc was greater in the 30 than the 6 hour group (4.14 % ± 1.27 % and 3.73 % ± 0.83 %) before declining thereafter.

2.4.5 Gamma camera assessed In-wbc influx

Figure 2-15 Neutrophil influx assessed by external scintigraphy

The graph shows the amount of neutrophil influx into the right, pneumonic lung at different time points in each type of pneumonia, as assessed by ante-mortem gamma scintigraphy. The graphs show means, error bars SEM, n=14 to 17 per group. The legend shows the significance of the effect of time for each group (ANOVA). * represents a significant difference from control at p <0.05.



Gamma camera images generally confirmed the pattern seen with tissue gamma well counts. No net influx of In-wbc was seen in control, unbronchoscoped animals. In fact, the correction produced an apparent 'negative' influx (mean -1.63 % $\pm$ 0.55 SEM). Possible reason for this are examined in the discussion section.

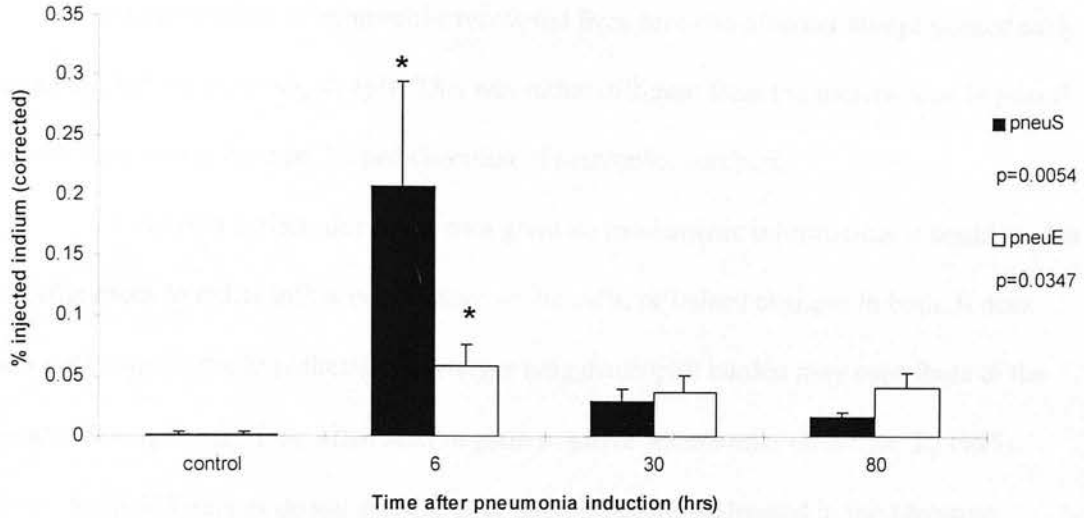
There was a significant effect of time in both pneuS and pneuE groups ($p = 0.0001$ and $p < 0.0001$). In pneuS, there was a significant increase in the 6 hour group (mean 5.37 % $\pm$ 1.46 SEM). In later groups there was a marked decline.

In pneuE, there was a significant increase compared to control not only in the 6 hour but also in the 30 hour group. In fact In-wbc in the 30 hour group (2.79 % $\pm$ 0.81) was almost as high as the 6 hour group (3.26 % $\pm$ 0.92) before declining.

2.4.6 In-wbc influx into BAL

Figure 2-16 Neutrophil influx into broncho-alveolar lavage fluid

The graph shows the amount of neutrophil influx into broncho-alveolar lavage fluid from the right, pneumonic lung at different time points in each type of pneumonia. The graphs show means, error bars SEM, n=14 to 17 per group. The legend shows the significance of the effect of time for each group (ANOVA). * represents a significant difference from control at $p < 0.05$.



There was hardly any In-wbc into broncho-alveolar lavage in control, unbronchoscoped animals (mean 0.001 % ± 0.003 %). There was a significant effect of time in both pneuS and pneuE ($p = 0.0054$ and 0.0347). In the pneuS there was a significant and marked increase at 6 hours (0.207 % ± 0.87), followed by a marked decline. In pneuE, there was also a significant increase at 6 hours (0.058 % ± 0.018). In later groups there was no longer a significant increase.

2.5 DISCUSSION

2.5.1 Histological appearances

The general light microscopic appearances of inflamed lung were not quantitatively examined. It was clear there was an intense inflammation of the affected lobe that

progressed from a neutrophil predominance to a mononuclear predominance before resolution commenced. Thus, the model produced the classical appearances of pneumonia. It was possible to confirm from macroscopic appearances that inoculates were consistently placed in the dorsal part of the right cranial lobe.

2.5.2 BAL neutrophil content

The total number of neutrophils recovered from broncho-alveolar lavage peaked early in pneuS, before declining steeply. This was rather different from the pattern seen in pneuE where there was a more prolonged elevation of neutrophil numbers.

As discussed earlier, this on its own gives no mechanistic information; it could be due to differences in either influx or clearance of the cells, or indeed changes in both. It does however support the hypothesis that a larger lung neutrophil burden may contribute to the greater damage to the lung often seen in gram negative pneumonias (Coalson, J., 1995).

Such differences do not seem to have been explicitly addressed in the literature. However, work as yet available only in abstract form (using tissue morphometry in a similar rabbit model) agrees that the number of neutrophils in the lung remains elevated for longer in pneuE than in pneuS (Kutkoski et al, 1996).

2.5.3 BAL alveolar macrophage content

In both types of pneumonia, total broncho-alveolar lavage recovery of alveolar macrophages remained unaffected until the 80 hour group (i.e. in broncho-alveolar lavage fluid obtained 101 hours after induction of pneumonia). A significant delayed elevation is consistent with classical observations of pneumonia (e.g. Loosli 1942a).

2.5.4 Influx of ^{111}indium labelled neutrophils into areas of pneumonia

This was assessed both *post mortem* by tissue gamma well counting and *ante mortem* by external gamma scintigraphy.

Using both methods, the pattern of influx observed was similar. In pneuS there was an initial marked increase in labelled cell influx, but this markedly declined at both later time points. In pneuE, neutrophil influx followed a different pattern, with neutrophil influx being as high in the 30 hour as the 6 hour time point.

The magnitude of the peak influx measured by the two methods was similar, lending validity to the measures. That they were not identical is to be expected on a number of accounts. There may be changes in blood pooling ante mortem, and the pulmonary vasculature was flushed before tissue counting. In addition, the entire lung was counted in the tissue gamma well counter. The region of interest studied on external scintigraphy only includes the more cranial portion of the lung, as the region of interest had to be constructed clear of the dome of the liver, which had significant activity. External scintigraphy was performed before, but tissue well counts after bronchoalveolar lavage. This would have removed some of the recruited cells. Rabbits with pneumonitis induced by C5f had only about 1 to 2 % of neutrophils present in the airspaces (as determined by morphometry) removed by broncho-alveolar lavage (Downey et al, 1993), so in fact differences due to this should be small.

Curiously, control rabbits had an apparent deficit of neutrophils in the lung when assessed by external scintigraphy. If Tc-rbc were detained in the pulmonary vasculature either because of aggregation or damage, this could produce such effects as the relative excess of ^{99m}Tc counts would give rise to over-correction. In addition, external scintigraphy was undertaken after 5 minutes of equilibration of erythrocytes, whereas tissue counting was performed after an hour, which could allow some redistribution. As discussed, the limited apparent volume of distribution of Tc-rbc indicates there was no gross retention. More likely is the effect of spillover between the ^{99m}Tc and ^{111}In windows on the gamma camera, which was not corrected for. As spillover was greater for ^{111}In to ^{99m}Tc windows than vice versa, correction of a highly vascular area such as the lung using data from less

vascular abdominal viscera could lead to over-correcting of blood volume. This would also tend to blunt the recruitment signal seen, as spillover of ^{111}indium activity would lead to overestimation of blood volume at the inflamed site. Tissue well counts did utilise a correction for spillover. Such spillover is not a linear function of activity, so correction factors are in themselves no guarantee of accuracy. Thus it was felt appropriate to utilise measures with and without correction and to compare them. That these technically disparate methods indicate similar patterns of response actually lends support to the overall result.

At maximal influx, around 5 % of labelled neutrophils were recruited to the pneumonic area. This compares quite favourably to the finding on morphometric and physiological measurement that only 1-2 % of neutrophils delivered to an area of pneumococcal pneumonia are actually recruited during the first 8 hours of disease (Doerschuk et al, 1994).

2.5.5 Influx of ^{111}indium labelled neutrophils into airspaces during pneumonia

Influx of ^{111}indium labelled neutrophils into the airspaces of lungs was assessed by measuring their accumulation in broncho-alveolar lavage fluid. PneuS produce a similar influx pattern to that seen with external gamma scintigraphy or tissue well counting. There was a high initial peak, with a rapid subsequent fall.

PneuE produced a slightly different pattern. There was significant elevation only at 6 hours, but by 30 hours this was non-significant, unlike the other measures of neutrophil influx which were significantly elevated in the 30 hour group too. This could demonstrate that at later time points more of the recruited neutrophils are interstitial, adhere more firmly or are otherwise less available to broncho-alveolar lavage. Indeed, broncho-alveolar lavage produced a mean of just 0.91 % of labelled cells compare to tissue well counts in this group compared to 1.51 to 3.86 % in other groups, even though histology shows the majority of neutrophil accumulation to be to the airspaces rather than the interstitium. These figures are

incidentally in good overall agreement with the morphometrically calculated recovery of 1 - 2 % of intra-alveolar neutrophils by broncho-alveolar lavage (Downey et al, 1993). The data from broncho-alveolar should be interpreted with some caution as it is potentially subject to greatest errors. The gamma counts are relatively low, so subject to more intrinsic variation. In addition, if inflammation is intense, the non-compliant lung tissue will divert lavage fluid to more compliant areas of the lung. In pneuE, 30 hour ^{111}indium labelled neutrophil influx into BAL was actually not much less than at 6 hours, and its failure to reach significance statistically may be misleading.

2.5.6 Comparison with other studies

A previous study demonstrated maximal neutrophil influx to pneuS between 2 and 6 hours, with none occurring after 24 hours, which is in broad agreement with this study (Jones et al, 1994). In a dog model of pneumococcal pneumonia, continuing neutrophil recruitment as assessed by 4 hour retention of ^{111}indium-oxine-labelled neutrophils in the lung was seen at 24 hours (Lichter et al, 1984). The large signal they saw is relatively late compared to this study. However, they instilled pneumococci in arrowroot in order to avoid its dispersion, and this may well have change the kinetics considerably.

There have been two clinical studies using ^{111}indium-labelled cells and gamma scintigraphy in patients with pneumonia. 5 patients with lobar pneumonia showed no excess pulmonary uptake, whereas patients with lung abcess did demonstrate pulmonary neutrophil influx (Saverymuttu et al, 1985). Patients in this study had symptoms between 2 days and 4 weeks prior to examination, so the lack of influx is perhaps not surprising, and is certainly compatible with the current findings.

In a study of patients with acquired immunodeficiency syndrome undergoing nuclear medicine investigations for fever, 10 patients had focal pulmonary uptake on either ^{111}indium-neutrophil scans or ^{67}gallium citrate scans (Fineman et al, 1989). 7 of these had a

final diagnosis of broncho-pneumonia, and of these five had evidence of neutrophil influx on In-wbc imaging. Unfortunately clinical details are sparse, and the timing of the scans in the disease is not clear. The positive scans seen in this study as opposed to those of Saverymuttu et al may be due to different timing, the fact that broncho-pneumonia rather than lobar pneumonia was studied, or to the fact that one study used patients who were HIV positive. Unfortunately the lack of detail does not allow firm conclusions to be drawn.

2.5.7 Mechanism of difference of neutrophil influx patterns

The mechanism leading to an end to the influx of cells to an inflammatory site is not clear. In an experimental arthritis model it appeared a loss of chemoattractant activity was important. Re-challenging the joint after an interval produced a similar response, with no evidence of loss of sensitivity to suggest production of an inhibitor or desensitisation of the recruitment process (Haslett et al, 1989). On the other hand, rechallenge of the lung with Pseudomonas aeruginosa within a week of an earlier challenge produces a much impaired neutrophil recruitment that seems to be associated with a local loss of ability to use the CD18-mediated path of adhesion, and the development of an alternative much less efficient pathway (Kumasaka et al, 1996). Whether the differences between the study relate to the longer time point examined in the latter study, differences due to stimuli used, or differences in the vascular beds studied is not at all clear.

Removal of the inciting stimulus may be important. Certainly in ongoing bronchial or intra-pulmonary sepsis there is ongoing neutrophil influx (Saverymuttu et al,1985, Currie et al, 1985). However, ongoing neutrophil recruitment may also be seen after intrapulmonary instillation of bleomycin, which is presumably rapidly cleared (Haslett et al, 1989). That the neutrophil response is not merely a function of the continued presence of a stimulus is suggested by further lines of evidence. In complement-deficient mice, clearance of Staphylococci from the lungs was unaffected, as was the neutrophil response. Impaired

clearance of pneumococci was correlated with a decreased neutrophil recruitment, whereas although Pseudomonas clearance was greatly impaired there was a normal neutrophil response (Hendrick et al, 1982). In addition, 13 different strains of Pseudomonas aeruginosa were found to have 4 different clearance patterns, with a variety of different histological patterns being produced. No clearance pattern could be related to production of a particular histological type (Southern et al, 1970).

It is notable that differences of bacterial persistence have been shown. 4 hours after aerosolisation, 7 % of Strep. pneumoniae within the lung were viable, compared to 89 % of E. coli (Jay et al, 1976). Using a combination of radiolabels to track mass clearance, quantitative culture, and tetracyclines to inhibit *in vivo* growth, they were able to show this was due to a combination of more rapid intrapulmonary growth and decreased killing of E coli compared to Strep. pneuomoniae.

These persistence studies were performed using nebulised bacteria. Using lower innoculates of bacteria administered as a bolus, marked proliferation of Strep. pneumoniae was seen over 24 hours(Ansfield et al, 1977).

In the experiments presented here, bacterial viability was not assessed, so its contribution cannot be directly judged.

The mechanism of the prolonged neutrophil influx demonstrated in pneuE compared to pneuS is thus unclear, although a contribution of increased persistence is possible.

2.6 SUMMARY

1. A rabbit model of localised pneumonia was established. This gave the expected pattern of inflammation, commencing with an inflammatory infiltrate that was initially neutrophilic but later was predominantly mononuclear, before resolution commenced. Recovery of cells from broncho-alveolar lavage showed the total neutrophil load was maximal in the 6 hour group and decreased thereafter. In pneuE, neutrophil numbers

remained high in the 30 hour group, imposing a greater overall neutrophil burden in pneuE than pneuS.

2. Neutrophils labelled with ^{111}indium-tropolonate were prepared to protocols that had previously been shown to produce cells with normal physiological functions. Cells used in these studies had a circulating half-life of around 10 hours, a little longer than, though roughly commensurate with, previous studies. Although there was some loss of binding of label over the course of the study, localisation to sites of inflammation was determined by external scintigraphy to be dependent on labelled cells, and label in the tissues was cell associated.

3). Autologous erythrocytes labelled with ^{99m}technetium had a short circulating half-life indicating some damage, but had a normal initial apparent volume of distribution, suggesting they were evenly distributed around the vasculature.

4) Using assessment by gamma scintigraphy or tissue gamma well counts, the neutrophil influx in pneuS was found to be high at 6 hours and decline thereafter. The influx in pneuE was maintained in the 30 hour group before falling.

5). Neutrophil influx into broncho-alveolar lavage fluid was similar in pneuS and pneuE. This apparent difference from other assessments may relate to a smaller number of cells in the airways being accessible to lavage at 30 hours in pneuE, or the greater inherent variability of lavage measures.

6). Overall, the more prolonged increase in total number of neutrophils in pneuE than pneuS is seen to reflect differences in influx patterns. The mechanism for this is uncertain, although some data suggests greater intrapulmonary survival of Strep. pneumoniae than E. coli. Although the more prolonged neutrophil influx demonstrated might then be regarded as an appropriate response to prolonged bacterial challenge, it is suggested it the increased lung neutrophil burden over time may give rise to the greater lung damage classically associated with gram negative pneumonia.

3. THE ROLE OF THE CD18 ADHESION MOLECULE IN NEUTROPHIL INFLUX IN PNEUMONIA

3.1 Introduction

In the lungs, neutrophils migrate specifically from the capillaries, as opposed to the post-capillary venules in the systemic circulation (Downey et al, 1993). Before they can accomplish this, circulating neutrophils must first adhere to the vascular endothelium.

As reviewed in the main introduction, selectins and β_2 integrins are thought to be of critical importance in migration from the systemic circulation (see Butcher, 1991, Furfaro et al, 1992, and Smith, 1992b). The situation in the pulmonary circulation is less clear cut. In pneumococcal pneumonia there is, for instance, doubt that any of the selectins or that β_2 integrins are involved in adhesion and migration at all (Doerschuk et al, 1990 and Mizgerd et al,1996), when other as yet undiscovered molecules are presumably important.

The variety of adhesion molecules available with their different characteristics and dynamics means that there is great potential for careful modulation of neutrophil influx *in vivo*. Progressive changes in the way adhesion molecules are controlled and utilised may be a crucial way in which the neutrophil influx increases, peaks and declines, and may be essential to the efficient termination of recruitment.

The expression of selectins on activated endothelial cells varies with time. P-selectin is rapidly translocated from intra-cellular stores in Wiebel-Palade bodies, appearing on the cell surface in minutes (McEver et al, 1989). E-selectin requires new protein synthesis for expression and as a consequence its appearance is delayed, being expressed maximally after 4 to 6 hours (Bevilaqua et al, 1989). Expression then decays so it is no longer expressed after 24 hours. The potential relevance of this *in vivo* is demonstrated by the finding that anti-P-selectin antibody blocks neutrophil accumulation in response to thioglycollate

induced peritonitis early in the disease, but only inhibits neutrophil accumulation later if E-selectin is absent (Labow et al, 1994).

The expression of ICAM-1, the immunoglobulin superfamily member that acts as the most important β_2 integrin counter-receptor on the endothelial cell, is constitutively expressed at low levels, but cell activation leads (via new protein synthesis) to increased expression. This takes several hours, but is maintained as long as activating factors are present in culture, and certainly beyond 24 hours (Dustin et al, 1989). An example of the potential *in vivo* relevance of this is provided by the finding that CD18-dependent neutrophil influx in response to LPS is associated with increased ICAM-1 expression on pulmonary capillary endothelium, whereas CD18-independent neutrophil influx in response to Strep. pneumoniae is not (Burns et al, 1994).

The regulation of neutrophil surface β_2 integrin molecules is rapidly modulated both by increased surface expression, by translocation of preformed molecules to the cells surface, and by functional changes in expressed molecules (see Dransfield, 1991). L-selectin is rapidly cleaved from activated neutrophils (Kishimoto et al, 1989).

The vast majority of studies of β_2 integrin function *in vivo* have examined its rôle very early in the inflammatory process, usually within 4 to 12 hours of its onset or even earlier. In the few studies continued beyond this time, there is certainly evidence that the requirement for β_2 integrins in neutrophil accumulation during inflammation changes with time. Instances of this have been found in studies of regions supplied by both the systemic circulation (Winn and Harlan, 1993) and the pulmonary circulation (Kumasaka, 1996). (Strictly, the latter actually examined change in use of β_2 integrins with time in recurrent pneumonia, rather than during the evolution of the process on a single occasion). However, both these studies measured total neutrophil numbers. As discussed, this may reflect either influx or clearance rate changes. As well as mediating cell adhesion, integrins participate in signalling information about the external environment into the cell (Erle and Pyetal, 1992),

and it is certainly conceivable that this could modulate neutrophil apoptosis and hence clearance. Thus, blocking CD18 function could have additional or alternative effects on neutrophil numbers in tissues than just those produced by inhibiting adhesion.

One study has used ^{111}indium labelled cells to examine the effect of anti-CD18 antibodies on neutrophil kinetics at different time points (McCandless et al, 1994). This study examined the response produced 4 or 24 hours after intravenous administration of LPS. They measured initial pulmonary retention and half-life of clearance of neutrophils from the lungs. Thus they were measuring a sequestration rather than a migration signal, but the finding that increased sequestration was CD18-dependent late (after 24 hours) but not early (after 4 hours) is interesting, particular as it is the opposite to the change in CD18 dependency seen in some models of peritonitis by Winn and Harlan. This apparent difference could arise for a number of reasons; a difference when measuring a sequestration rather than a tissue accumulation signal, a difference between types of stimuli used, a difference intrinsic to the pulmonary as opposed to the systemic circulation, or a difference between local and systemic administration of inflammatory stimulus.

Accumulation of neutrophils in the lung in pneuS is already known to be unaffected by the presence of anti-CD18 antibody in rabbits (Doerschuk et al, 1990). Doerschuk et al found anti-CD18 to significantly inhibit neutrophil accumulation over 4 hours in response to LPS instilled intrabronchially, but this was not examined thereafter. Attention was therefore focused in this study on pneuE. Neutrophil influx was specifically examined by measuring the accumulation of donor ^{111}indium labelled cells (as in the experiments described in chapter 2) at 6 and 30 hours. The inflammatory process was allowed to proceed normally until the injection of the anti-CD18 antibody, 6.5e, 15 minutes prior to administration of labelled cells. Thus, any change in CD18 dependency would be unlikely to be due an induced change in the inflammatory process secondary to the CD18 blockade produced by the antibody.

3.2 Methods

The antibody used for these experiments, 6.5e, was kindly donated by Celltech Ltd., Slough. It is an anti-human CD18 antibody known to cross react with rabbit CD18 (Galea-Lauri et al, 1993), and is a monoclonal mouse IgG1. It was supplied in PBS at a concentration of 2.4 mg/ml, and contained 39 Eu/ml of endotoxin. In previous studies in the guinea pig, 2.4 mg/kg were shown to significantly inhibit neutrophil recruitment to the skin in response to zymosan activated palsma (Teixera, M. M. et al, 1994). Whole animal studies using 6.5e to inhibit neutrophil recruitment in rabbits suggest 1 mg/kg causes significant inhibition of neutrophil recruitement (M. Robinson, personal communication). Preliminary experiments were performed to check that neutrophil surface CD18 saturation was achieved.

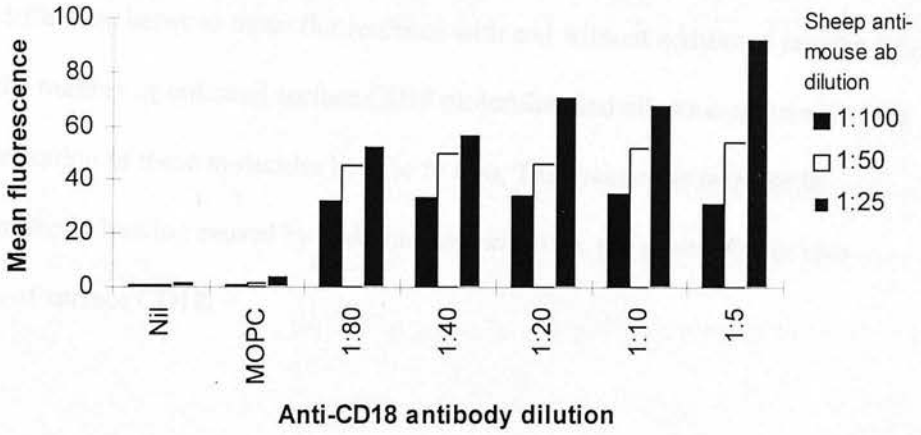
3.2.1 In vitro *assessment of antibody binding*

Rabbit neutrophils were placed in a round-bottomed 96 well polypropylene flexiwell plate at 2 x 10^5 per well. Cells were pre-incubated on ice with 100µl of 10 % normal rabbit serum (from donor rabbits) in assay buffer (PBS with cations, 0.2 % (w/v) BSA and 0.1 % (w/v) sodium azide). They were washed with 100µl of assay buffer, followed by buffer only, MOPC (control antibody, derived from ATCC clone, courtesy I. Dransfield, Rayne Laboratory, Edinburgh) to a dilution of 1:25 in assay buffer, or 6.5e to a dilution of 1:80, 1:40, 1:20, 1:10 and 1:5 were add to six wells each. These mixtures were incubated for 30 minutes on ice, before being washed twice with assay buffer. Sheep anti-mouse IgG-FITC conjugated antibody (α-m FITC) (Scottish Antibody Production Unit, Carluke) was added in assay buffer to a dilution of 1:100, 1:50 and 1:25, each in duplicate for each primary antibody condition. These were incubated on ice for 30 minutes, then washed twice with assay buffer. Neutrophils were then fixed with 100µl of 2 % (v/v) formaldehyde (BDH Laboratory Supplies, Poole). A Coulter flow cytometer (Epics Profile II, Coulter

Electronics, Luton) was used to assess antibody binding. Neutrophils were gated on using forward and side scatter characteristics, and results expressed as mean fluorescence.

Figure 3-1 In vitro binding of 6.5e

Isolated rabbit neutrophils were treated with either a control antibody (MOPC) or ascending doses of 6.5e as primary antibody, with sheep anti-mouse FITC as secondary antibody at one of three dilutions. Mean fluorescence is plotted, with data being the mean of duplicates in a single experiment.



PBS and MOPC controls resulted in virtually no neutrophil associated fluorescence, except with the highest concentration of α-m FITC, when a little non-specific binding began to be apparent. Antibody 6.5e resulted in a marked increase in fluorescence, even at a dilution of 1:80, indicating a marked specific binding to neutrophils.

3.2.2 In vivo *antibody binding*

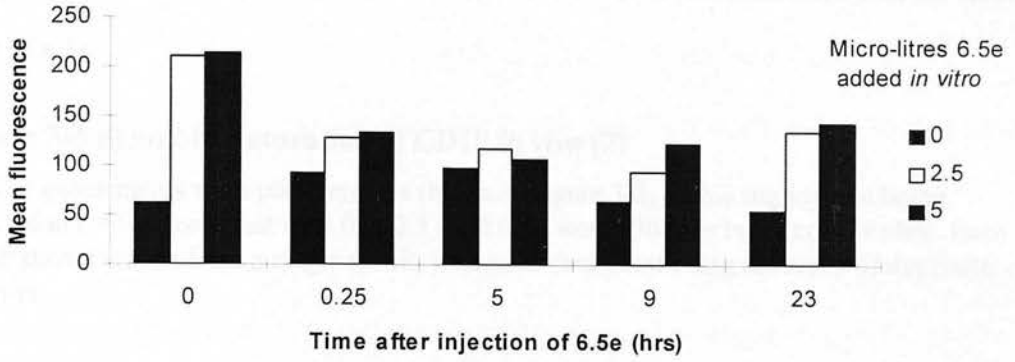
Varying regimes of 6.5e dosage were used, as indicated below. Before and at intervals after injection of 6.5e, 2 ml blood samples were collected into tubes containing EDTA anticoagulant and immediately transferred to ice. These were centrifuged at 300g for 6 minutes at 4°c. The buffy coat was aspirated using a transfer pipette and washed with normal saline. In the first experiment, the cell pellet was then split between 6 wells of a 96 well

flexiwell plate, and processed as for the assessment of *in vitro* antibody binding. Duplicate wells had either no antibody, or 1:40 or 1:20 dilutions of 6.5e added as primary antibody. 1:50 dilution of α-m FITC were then used as secondary antibody. Gating on neutrophils during flow cytometry eliminated contaminating erythrocytes, lymphocytes and monocytes and allowed mean fluorescence of neutrophils to be measured. In the first experiment, no significant difference in mean fluorescence was obtained between samples after the addition of 1:40 or 1:20 dilutions of 6.5e, so further experiments used only 1:40 dilutions.

The difference between mean fluorescence with and without additional *in vitro* 6.5e represents the number of unbound surface CD18 molecules, and allows assessment of the degree of saturation of these molecules by 6.5e *in vivo.* The greater the increase in secondary antibody binding caused by additional *in vitro* 6.5e, the greater the *in vivo* desaturation of surface CD18.

Figure 3-2 Saturation of CD18 *in vivo* (1)

Blood samples were taken immediately prior (t = 0 hrs) and at intervals following the administration of 1 mg/kg 6.5e to a rabbit. Either 0, 2.5 or 5 µl of additional 6.5e were added *in vitro* to this, and the presence of bound 6.5e detected by sheep anti-mouse FITC antibody. The graph shows mean of two paired samples for each data point in a single experiment.



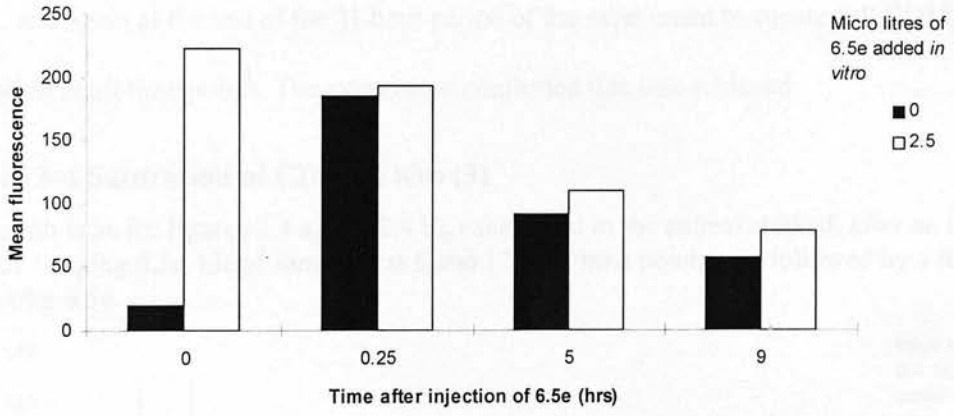
The first such experiment showed that before injection of 6.5e, there was some background binding of α-m FITC. Addition of 2.5 µl or 5 µl of 6.5e *in vitro* resulted in a marked increase in binding of α-m FITC, showing there were large numbers of exposed CD18, as expected. There was little difference in binding produced with either amount of added antibody, showing the lower amount was saturating even for control cells, and this was used in subsequent experiments. At 15 minutes and 5 hours after injection of 6.5ebinding of α-m FITC to buffy coat neutrophils in the absence of any *in vitro* addition of 6.5e was increased, showing that 6.5e was binding *in vivo.* However, there was a small increase produced by the addition of 6.5e *in vitro*, showing binding was not saturated *in vivo.* By 9 and 23 hours, α-m FITC binding to buffy coat neutrophils without additional 6.5e returned to control levels. It was markedly enhanced by the addition of further 6.5e showing a large degree of desaturation. It was notable that at all time points, the maximum amount of α-m FITC binding produced by saturating amounts of 6.5e was less than in control cells, demonstrating that the total surface expression of CD18 by neutrophils was depressed by injection of 6.5e. This is presumably secondary to receptor crosslinking and internalisation.

It was surprising the binding seen was not more marked, in view of the fact that similar doses had produced significant effects on neutrophil migration in other models (M. Robinson, personal communication), and had usually been found to be saturating in a variety of animal models. Two further similar experiments were therefore performed with the same dose of 6.5e.

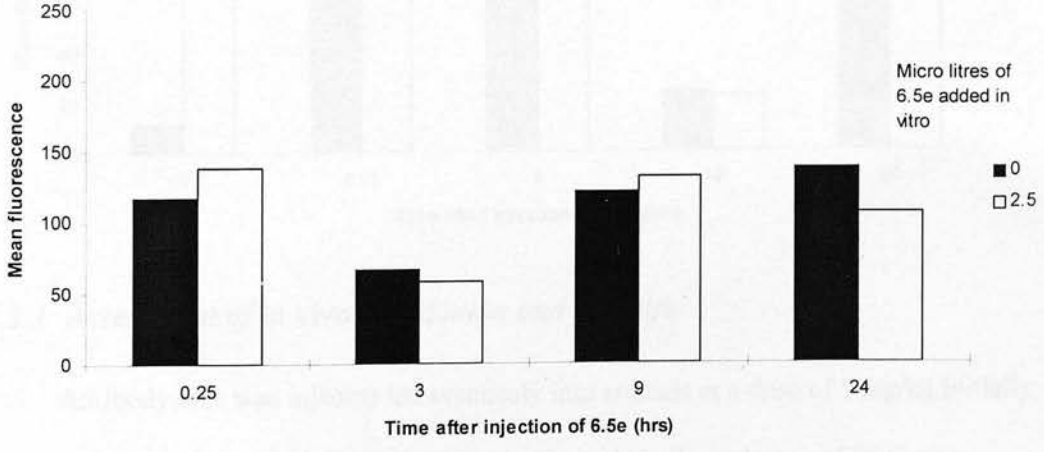
Figure 3-3 a) and b) Saturation of CD18 *in vivo* (2)

Similar experiments were performed as shown in Figure 3-2, with 1 mg/kg 6.5e being injected at t = 0, except that only 0 or 2.5 ul of 6.5e were added to buffy coat *in vitro.* Each graph shows results from a single rabbit, with each data point being the mean of duplicate samples.

a)



b)



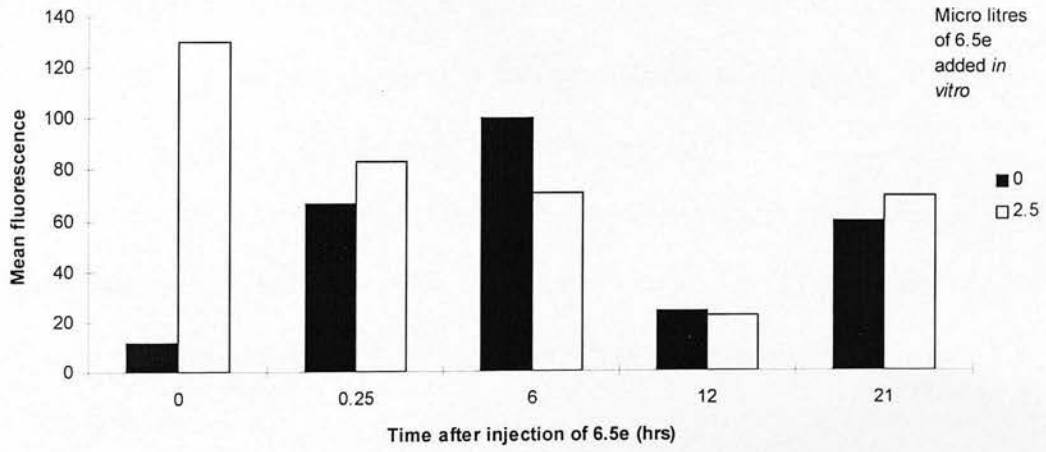
The first experiment showed almost complete saturation of CD18 to the end of the experiment at 9 hours. The second experiment was extended to 24 hours and showed

virtually complete saturation throughout. The experiments also showed the previously demonstrated decrease in total surface CD18 expression after 6.5e administration *in vivo.*

Thus, two out of three experiments showed 1 mg/kg of 6.5e to saturate neutrophil CD18 *in vivo.* The third experiment did not show saturation to be complete, and showed little binding of 6.5e to surface CD18 after 9 hours. Therefore the regime of 6.5e administration selected for the main experiments was 1 mg/kg initially to ensure immediate saturation of CD18, followed 6 and 12 hours later by doses of 0.5 mg/kg. A final experiment was performed to ensure CD18 saturation by 6.5e throughout the 21 hours of the experimental. Blood samples were taken immediately prior to injection of 6.5e at 6 and 21 hours, and again at the end of the 21 hour period of the experiment to ensure full CD18 saturation at all time points. The experiment confirmed this was achieved.

Figure 3-4 Saturation of CD18 *in vivo* (3)

The graph is as for figures 2.4 a) and 2.4 b), except that in the animal studied, after an initial dose of 1 mg/kg 6.5e, blood sampling at 6 and 12 hour time points was followed by a further 0.5 mg/kg 6.5e.



3.2.3 Assessment of in vivo *distribution and half-life*

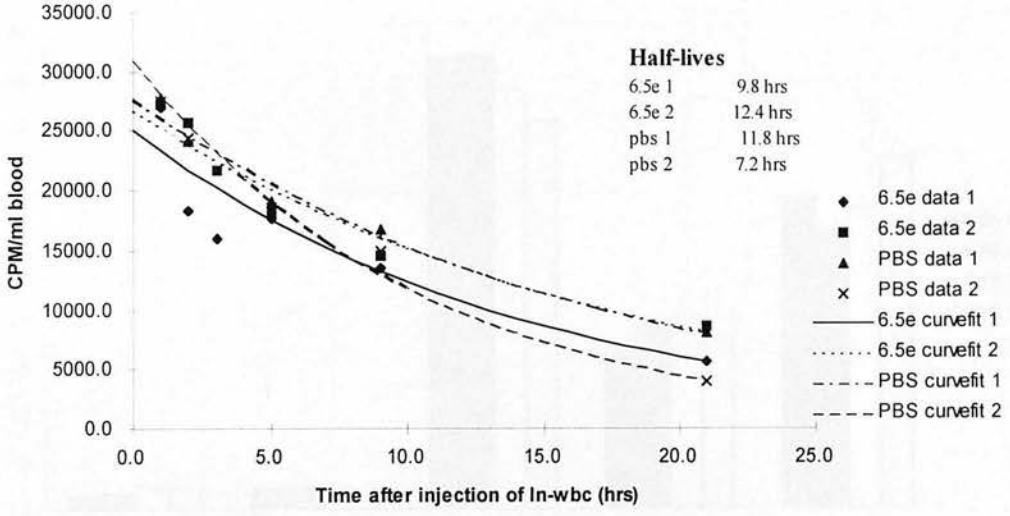
Antibody 6.5e was injected intravenously into animals at a dose of 1 mg/kg initially and at 0.5 mg/kg at 6 and 12 hours in two animals, and similar volumes of PBS were injected into two further animals as controls. 5 minutes after the initial injection of 6.5e or PBS, ^{111}indium labelled neutrophils prepared as described were injected intravenously. Two

ml arterial blood samples were taken into tubes with EDTA anti-coagulant at intervals thereafter, and immediately transferred to ice. Samples were weighed to allow correction for volume. They were centrifuged at 300g for 6 minutes, and plasma aspirated from cells. 5 ml of normal saline was added, and the blood was re-centrifuged and the cell-free supernatant aspirated. Gamma activity of the cells was determined using a tissue gamma well counter. An exponential curve was fitted to variation of cell bound activity with time. The half-life of labelled cells was determined as described in the previous chapter.

21 hours after injection of ^{111}indium-labelled neutrophils, their distribution was assessed using gamma scintigraphy. Lung regions of interest were constructed as described, whilst liver and spleen regions of interest were drawn freehand. The remaining activity (predominantly in the blood pool and the marrow) was calculated by subtraction of the other ROI's from the total.

Figure 3-5 Effect of 6.5e on In-wbc half-life

The graph shows cell-associated ^{111}indium per ml. of blood sample at intervals in four rabbits after injection of labelled cells. In two of these, administration of labelled cells was preceded by administration of 6.5e 1mg/kg, followed by 0.5 mg/kg at 6 and 12 hours. The other two received PBS control. Monoexponential curves are shown fitted to the data, and from these the half-lives of the injected neutrophils were calculated giving the results shown in the panel.

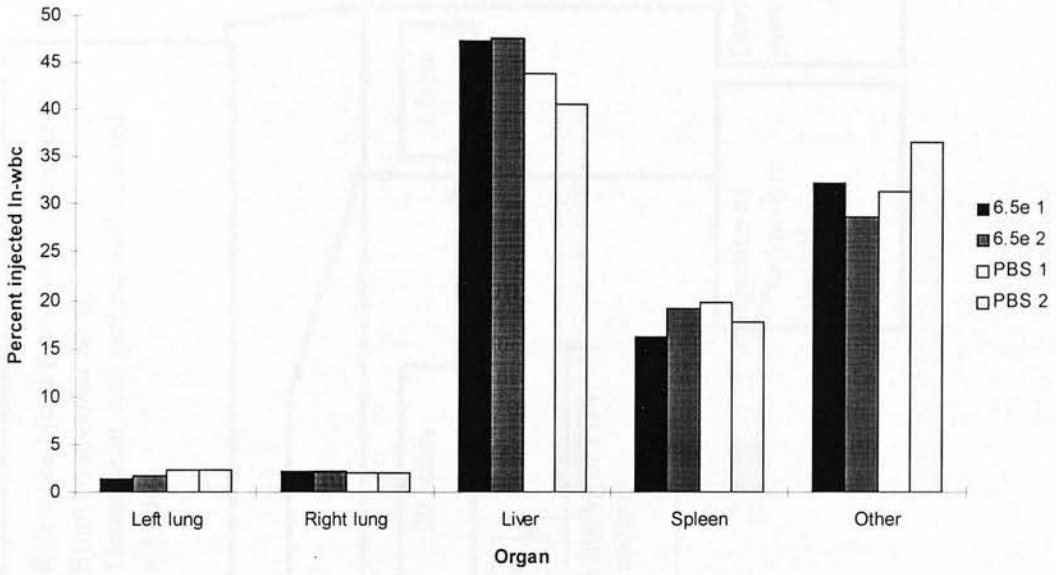


Counts obtained in four animals studied produced good exponential fits in all cases (F = 76.77, 69.71, 247.21 and 297.93, p = 0.003, 0.004, 0.001 and 0.001). Half-lives for clearance of In-wbc calculated using the method above showed values of 9.8 and 12.4 hours after 6.5e administration and 11.8 and 7.2 hours after PBS control.

The overall pattern of distribution of In-wbc was very similar in animals that had received 6.5e and PBS. In particular, the proportion of In-wbc in the right lung was similar in 6.5e and PBS treated animals (2.22 and 2.11 % in 6.5e treated and 2.04 and 2.10 % in PBS treated animals).

Figure 3-6 The effect of 6.5e on neutrophil distribution *in vivo*

The graph shows the percentage of ^{111}indium-labelled neutrophils in various organs 21 hours after injection, as determined by gamma scintigraphy, for four animals. In two cases animals received injections of 6.5e at the doses of 1, 0.5 and 0.5 mg/kg at 0 (prior to labelled cell administration), 6 and 12 hours respectively, and in the other two cases animals were administered equal volumes of PBS at these times.



3.2.4 Overall experimental plan

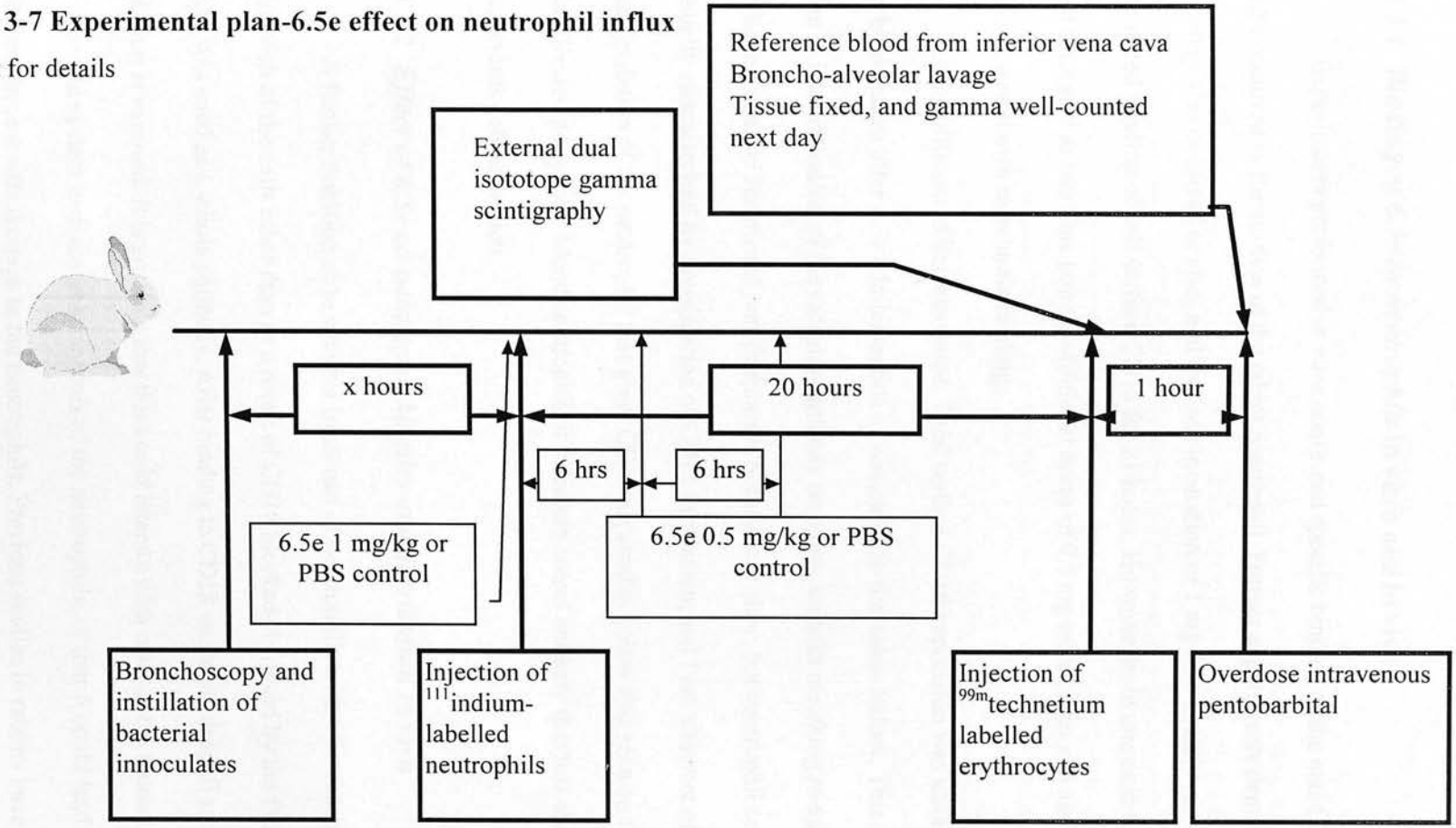
The experimental plan was based on that for 6 and 30 hour influx studies of pneuE, as described in chapter 2, except for the additional use of 6.5e or PBS control. Animals were treated as pairs, with each receiving ^{111}indium-labelled neutrophils and E. coli suspension from the same pools. 5 minutes prior to the injection of ^{111}indium-labelled neutrophils, one rabbit received 1 mg/kg of 6.5e intravenously, and 6 and 12 hours later received further aliquots of 0.5 mg/kg. The other received equivalent volumes of PBS as control.

3.2.5 Presentation of results

Neutrophil influx data was calculated as described in chapter 2. Paired t-tests were used to assess significance of differences between treated and untreated animals within groups.

Figure 3-7 Experimental plan-6.5e effect on neutrophil influx

See text for details



3.3 Discussion of methods

3.3.1 Binding of 6.5e by neutrophils in vitro *and* in vivo

Experiments performed *in vitro* confirmed specific binding of the anti-CD18 molecule 6.5e occurred to the surface of the rabbit neutrophil. Further experiments demonstrated that binding also occurred *in vivo,* and that administration of 1 mg 6.5e usually resulted in saturated binding of cell surface CD18 for 21 hours. However, as in one case binding was not saturated at late time points, additional doses of 0.5 mg were given at 6 and 12 hours, which was shown to saturate binding.

An additional effect was noted. Total surface CD18 expression was usually depressed in blood taken after 6.5e administration as compared to that taken before. This is likely to be due to internalisation of the receptor/antibody complex, without matching re-expression. Whether this has functional ramifications is not entirely clear, but neutrophil activation is usually characterised by upregulation of CD18 expression, and I am unaware of any other manipulation of the neutrophil that gives CD18 expression below that seen on the unactivated peripheral blood neutrophil. It therefore seems unlikely that this change represents cell activation.

3.3.2 Effect of 6.5e on neutrophil kinetics and distribution in vivo

A further question as to whether treatment of neutrophils with 6.5e changes the function of the cells other than as a result of CD18 blockade is raised by the fact that the 6.5e was used as a whole antibody. After binding to CD18 on the neutrophil surface, its Fc portion is exposed. It is possible that this could interact with cells of the mononuclear-phagocyte system to cause phagocytosis of the neutrophils, or that it could lead to activation of complement with damage to the neutrophils. Previous studies in rabbits have not

suggested this to be the case, although a slight decrease in circulating neutrophil count has been observed in guinea pigs (M. Robinson, personal communication).

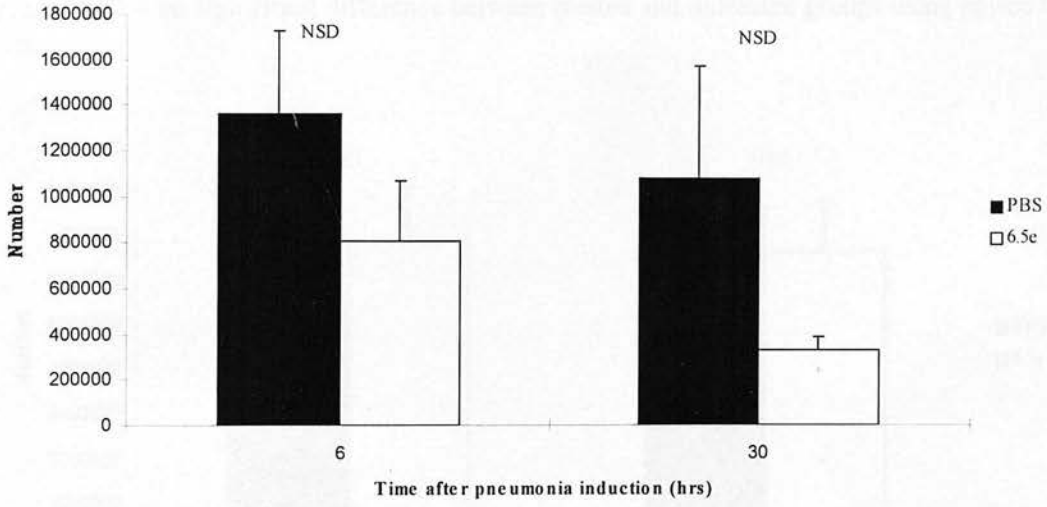
This was investigated further by comparing the circulating half-life of ^{111}indium labelled neutrophils in animals treated with 6.5e or PBS as control. No difference in half-life was shown in this sensitive test of neutrophil integrity. In addition, gamma scintigraphy of these animals demonstrated that the neutrophil distribution between each lung, the liver, the spleen and elsewhere (predominantly the blood and marrow) was unchanged by 6.5e treatment. Very subtle differences in treatment during ^{111}indium labelling of neutrophils leads to significant differences in *in vivo* kinetics and distribution (Saverymuttu et al, 1983). This clearly shows 6.5e has no non-specific detrimental effect on the neutrophil, and that following its administration, equal numbers of neutrophils will be available in the blood stream to be recruited as in controls.

3.4 Results

3.4.1 BAL neutrophil recovery

Figure 3-8 The effect of 6.5e on neutrophil recovery from broncho-alveolar lavage

The graph shows the total number of neutrophils recovered from broncho-alveolar lavage fluid from the right lung of rabbits with pneuE. Rabbits in 6 hour and 30 hour groups, treated with 6.5e or PBS control, are shown. Data shows mean, error bars SEM, n = 7 per group. NSD = no significant difference between treated and untreated groups using paired t tests.



There was no statistically significant difference in total neutrophil recovery from bronchoalveolar lavage induced by pneuE between animals treated with 6.5e and with PBS. There was a clear trend to inhibition of neutrophil recovery in response to 6.5e treatment in both groups.

Historical data from unbronchoscoped controls was used to calculate mean inhibition as for this and other data categories as follows:

Overall inhibition of neutrophil influx (per cent) = I
Neutrophil numbers in pneuE with 6.5e treatment = $PMN_{6.5e}$
Neutrophil numbers in pneuE with control treatment = PMN_{PBS}
Neutrophil numbers unbronchoscoped controls = PMN_{cont}

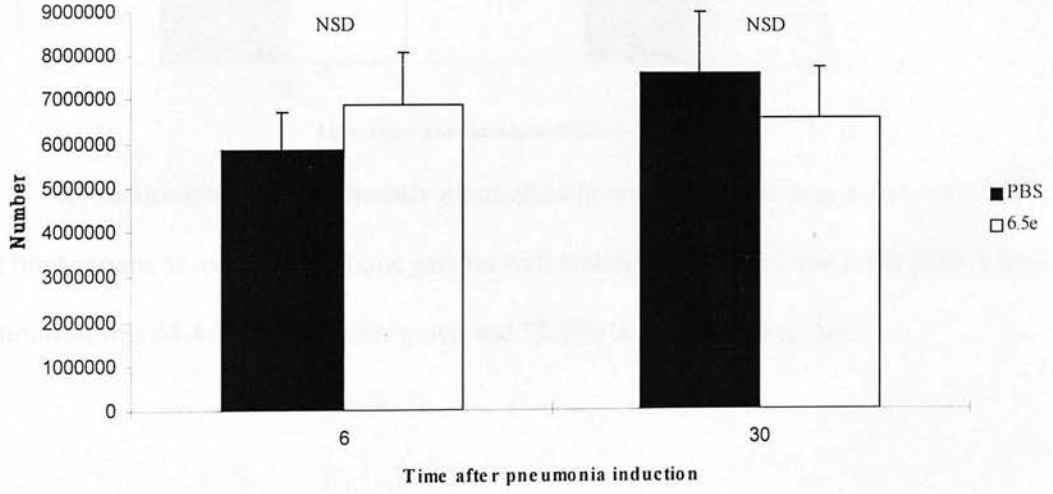
$$I = \left(1 - \frac{[PMN_{6.5e} - PMN_{cont}]}{[PMN_{PBS} - PMN_{cont}]}\right) \times 100$$

Calculated thus, mean inhibition of total neutrophil recovery in the 6 hour group (i.e. at 27 hours) was 42.2 % and mean in the 30 hour group (i.e. at 36 hours) was 71.8 %, indicating the trend towards inhibition of neutrophil recovery by 6.5e.

3.4.2 BAL alveolar macrophage recovery

Figure 3-9 The effects of 6.5e on alveolar macrophage recovery from broncho-alveolar lavage

The graph shows the total number of alveolar macrophages recovered from broncho-alveolar lavage fluid from the right lung of rabbits pneuE. Rabbits in 6 hour and 30 hour groups, treated with 6.5e or PBS control are shown. Data shows mean, error bars SEM, n = 7 per group. NSD = no significant difference between treated and untreated groups using paired t tests.

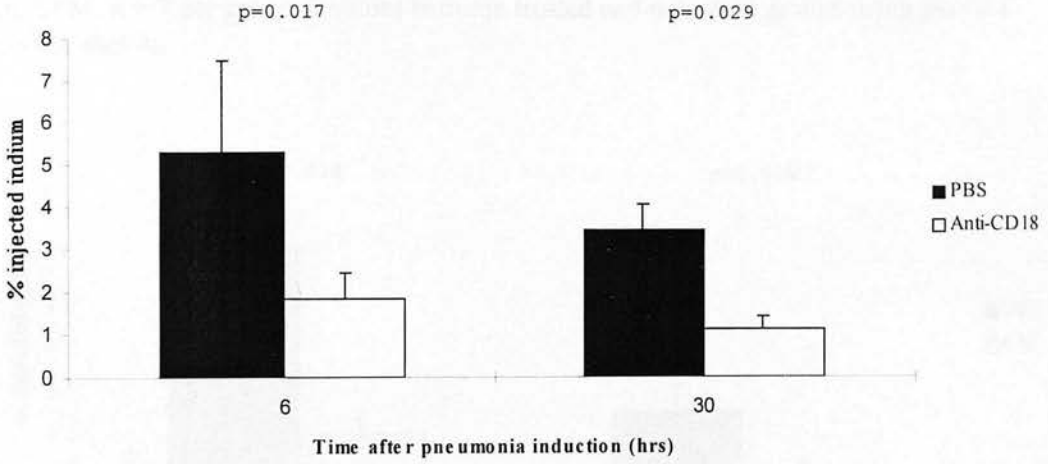


The total recovery of alveolar macrophages from broncho-alveolar lavage in pneuE was unaffected by 6.5e in both the 6 and 30 hour groups.

3.4.3 In-wbc influx as assessed by tissue gamma well counting

Figure 3-10 The effects of 6.5e on neutrophil influx as assessed by tissue gamma well counts

The graph shows the influx of neutrophils into the right lungs of rabbits with pneuE as assessed by corrected tissue ^{111}indium counts. Rabbits in 6 hour and 30 hour groups, treated with 6.5e or PBS control are shown. Data shows mean, error bars SEM, n = 7 per group. P values between treated and untreated groups using paired t tests are shown.

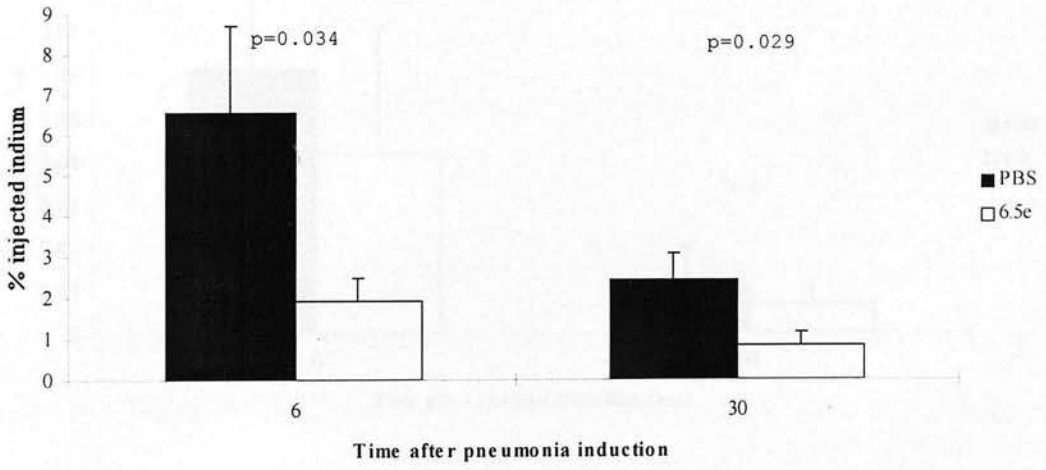


6.5e administration significantly diminished In-wbc influx into pneuE in both 6 and 30 hour groups as assessed by tissue gamma well counting ($p = 0.017$ and $p = 0.029$). Mean inhibition was 68.4 % in the 6 hour group and 72.7 % in the 30 hour group.

3.4.4 In-wbc influx as assessed by external gamma camera imaging

Figure 3-11 The effects of 6.5e on neutrophil influx as assessed by external gamma scintigraphy

The graph shows the influx of neutrophils into the right lungs of rabbits with pneuE as assessed by corrected ^{111}indium counts on external gamma scintigraphy. Rabbits in 6 hour and 30 hour groups, treated with 6.5e or PBS control are shown. Data shows mean, error bars SEM, n = 7 per group. P values between treated and untreated groups using paired t tests are shown.

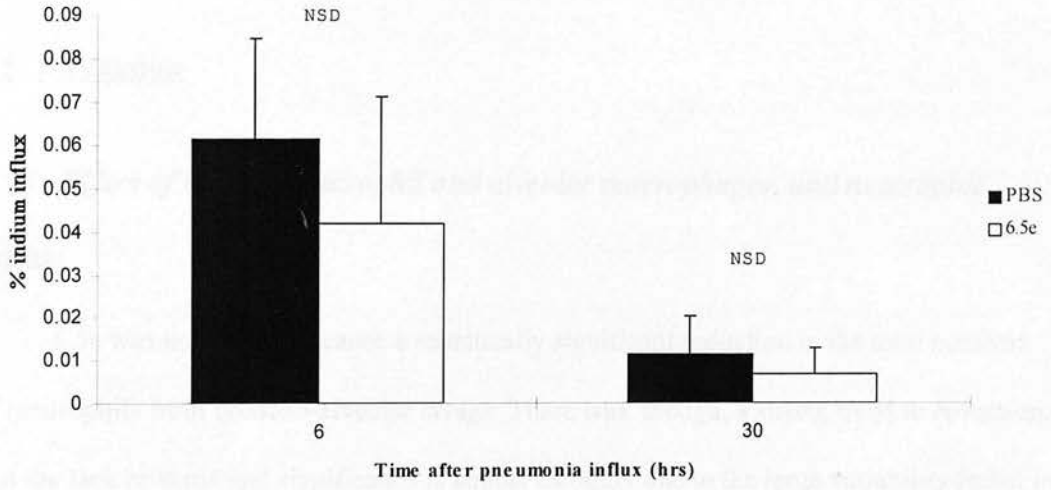


6.5e administration significantly diminished In-wbc influx into pneuE in both 6 and 30 hour groups as assessed by external gamma camera imaging ($p = 0.034$ and $p = 0.029$). Mean inhibition was 56.6 % in the 6 hour group and 39.2 % in the 30 hour group.

3.4.5 In-wbc influx as assessed by broncho-alveolar lavage well counts

Figure 3-12 The effects of 6.5e on neutrophil influx as assessed by gamma well counts of broncho-alveolar lavage fluid

The graph shows the influx of neutrophils into the right lungs of rabbits with pneuE as assessed by corrected ^{111}indium counts on gamma well counting of broncho-alveolar lavage fluid. Rabbits in 6 hour and 30 hour groups, treated with 6.5e or PBS control are shown. Data shows mean, error bars SEM, n = 7 per group. NSD = no significant difference between treated and untreated groups using paired t tests.



When In-wbc influx in response to pneuE was assessed by gamma well counting of bronchoalveolar lavage, there was no significant difference between 6.5e and PBS control treated animals, either in 6 or in 30 hour time points. The response was very variable, but there was a trend to inhibition of In-wbc influx. Mean inhibition of influx in the 6 and 30 hour groups was 31.7 % and 49.1 %.

3.4.6 Results of studies of the effect of 6.5e on neutrophil influx to pneuE - conclusions.

Neutrophil influx into pneuE was significantly (though not completely) inhibited by the anti-CD18 antibody 6.5e, as assessed by the accumulation of In-wbc measured by either

tissue gamma well counting or external scintigraphy. This was as true in the 30 as the 6 hour group, varying measures demonstrating between 50 and 70 % inhibition.

Neither total neutrophil numbers recovered by broncho-alveolar lavage, nor neutrophil influx into the airspaces as assessed by In-wbc accumulation into broncho-alveolar lavage showed significant inhibition by 6.5e. Both showed a trend to inhibition by 6.5e, but in both cases the magnitude (approx. 30-50 %) was rather less than the inhibition of In-wbc influx as assessed by tissue gamma well counts and external gamma scintigraphy.

3.5 Discussion

3.5.1 Effect of 6.5e on neutrophil and alveolar macrophages, and neutrophil influx

6.5e was not shown to cause a statistically significant reduction in the total numbers of neutrophils from broncho-alveolar lavage. There was, though, a strong trend to reduction, and the lack of statistical significance is almost certainly due to the large variability found in the results. As noted in the discussion of chapter one, only about 1 to 2 % of airspace cells are recovered from broncho-alveolar lavage, which may lead to significant variability. This is likely to be especially true in non-uniform inflammation. Inflamed areas of the lung are less compliant, and fluid will be diverted away from the inflamed area. Variability of access of fluid to the inflamed site may create sampling errors. In addition, intra-alveolar cells are likely to be not entirely free and separate, but bound to one-another and to epithelial cells. β_2 integrins may well be important in this, and penetration of 6.5e into inflamed areas may affect this binding. Thus, sampling by broncho-alveolar lavage may be less consistent in the presence of anti-CD18. In addition, it should be emphasised that the 6.5 e was only present during part of the duration of the experiment. In the 6 hour group, it was not present for the first 6 hours, but was present from hour 6 to hour 27. In the 30 hour group, it was not

present for the first 30 hours, but was present from hour 30 to hour 51. Particularly in the latter group, this will lead to a masking of any modulation of influx in terms of total neutrophil numbers. In view of all these factors it is not surprising that the antibody does not produce statistically significant decreases in lavage neutrophil number. The clear trend to inhibition is certainly not inconsistent with the hypothesis that anti-CD18 antibody reduces neutrophil influx.

6.5e, as expected, produced no effect on alveolar macrophage number in broncho-alveolar lavage.

Both *ante mortem* external gamma scintigraphy and *post mortem* tissue gamma well counting showed neutrophil influx to be inhibited by 6.5e in both 6 hour and 30 hour groups. Both methods showed inhibition to be around 60 % in the 6 hour groups. However, external scintigraphy showed an inhibition of neutrophil influx by 39.2 % whilst tissue gamma well counting showed an inhibition of neutrophil influx by 72.7 %. As noted in the discussion to chapter one, technical differences in the two methods as well as the fact one method uses *ante mortem* and one *post mortem* assessment make exact concordance unlikely. Overall it is clear that neutrophil influx is significantly inhibited, though not completely blocked, in both the 6 and 30 hour groups. The combined average inhibition found by the two methods in the 6 hour group was 62.5 % and in the 30 hour group was 55.9 %. These very similar values do not support the hypothesis that there is a significant change in the relative utilisation of β_2 integrins during pneuE.

Influx of neutrophils into broncho-alveolar lavage fluid as assessed by gamma well counting showed no statistically significant inhibition by 6.5e. There was a non-significant trend for inhibition in both time groups. As discussed above, measurements of cells recovered from broncho-alveolar lavage are intrinsically more likely to show variability than tissue counts, especially when anti-CD18 antibodies are used. In addition, relatively low levels of gamma activity were being counted in broncho-alveolar lavage compared to tissue,

again leading to greater intrinsic variability in the method. It is likely that the lack of statistically significant inhibition demonstrated using broncho-alveolar lavage fluid is due to this variability of results, and the trend of the results (mean inhibition of neutrophil influx of 31.7 % and 44.7 % in 6 and 30 hour groups) would certainly support this contention. The alternative explanation is that neutrophil accumulation in tissues is inhibited by CD18, but that a second CD18-independent mechanism exists for the transfer of cells from tissue to airspaces, and that this second process is saturated even with the decreased tissue neutrophil accumulation after CD18 blockade. This seems unlikely, particularly as the majority of accumulated neutrophils are seen histologically within the airspaces rather than the interstitium.

3.5.2 Comparison with other studies

Previous study of LPS instillation (rather than instillation of live E. coli) into the lung produced a neutrophil influx over the following 4 hours in rabbits. Pre-treatment with an anti-CD18 antibody produced a mean decrease of intra-alveolar neutrophils assessed morphometrically of 72 %. Over such short time periods this is very likely to represent an inhibition of neutrophil influx rather than survival, especially given the fact that normal lungs possess very few intra-alveolar neutrophils (Doerschuk et al, 1990). This partial inhibition observed is of a very similar magnitude to that seen in the current study. Doerschuk et al also showed that the increased number of intra-alveolar neutrophils 4 hours after intra-bronchial instillation of PMA was almost completely abolished by treatment with anti-CD18 antibody, suggesting that in pneumonitis due to LPS (and presumably in pneuE too) there is a CD18-independent as well as a CD18-dependent mechanism of recruitment. In the same series of experiments, neutrophil influx in response to hydrochloric acid or Strep. pneumoniae were found to be entirely CD18-independent.

The effect of anti-CD18 antibody on neutrophil influx (as assessed by accumulation of ^{111}indium-labelled neutrophils) in response to intra-bronchial C5a or IL-1α has also been investigated (Hellewell et al, 1994). Influx in response to C5a could not be significantly inhibited by anti-CD18 antibody, whereas that due to IL-1α could, although this inhibition was incomplete. Generation of both of these inflammatory mediators would be expected during pneumonia and they may thus contribute to the induction of the CD18-independent influx (and also, in the case of IL-1α, to CD18-dependent influx too) observed.

As discussed in the introduction to this chapter, the pulmonary sequestration of ^{111}indium-labelled neutrophils in response to intravenous LPS was found to be CD18-independent at 4 hours, but CD18-dependent at 24 hours (McCandless et al, 1994). It would be surprising if the mechanism of the sequestration of neutrophils changed without this being reflected in a change in neutrophil influx, as neutrophils must be sequestered prior to migration. The apparent contradiction between McCandless et al's findings and the current study are likely to be due instead to the systemic rather than local administration of inflammogen, and the fact that LPS was used rather than live E. coli.

In the rabbit peritoneum, the effect of anti-CD18 antibody on the number of neutrophils recovered from peritoneal lavage 4 or 24 hours after instillation of protease peptone or live E. coli was assessed in rabbits (Winn, R. K. and Harlan, J. M., 1993). Accumulation of neutrophils was found to be predominantly CD18-dependent at 4 hours, but was CD18-independent at 24 hours. Once again, it should be emphasised that these were measurements of total neutrophil number rather than specifically of influx, and caution should be exercised in equating the two.

In addition, in the study of Winn and Harlan, anti-CD18 antibody was administered prior to the induction of the inflammatory response. It is possible that over the next 24 hours inflammation was induced to proceed via an abnormal pathway. The development of CD18-independence at 24 hours could be viewed as an adaptive response to CD18 blockade rather

than a natural progression of the inflammatory process. In fact, they found that although the total number of neutrophils present at 4 hours was decreased, at 24 hours it was unchanged. Hence, there had been some 'catch up', implying there was indeed an adaptive response of some nature to CD18 blockade.

It is unclear whether the differences between the current study and that of Winn and Harlan relate primarily to difference in inflammatory site, or to differences between measuring neutrophil influx and total numbers, or differences between inflammatory processes modulated early in their evolution or allowed to develop naturally. Although other studies explicitly demonstrate differences between CD18 utilisation in areas supplied by systemic and pulmonary circulations and make this a perfectly plausible explanation, the 'catch up' in neutrophil numbers after 24 hours of CD18 blockade in the peritoneum raise serious question as to whether the blockade induced alternative pathways. This clearly has important implications for the possible therapeutic use of CD18 blockade as an anti-inflammatory manoeuvre.

Experiments have suggested that macrophages may be important in the induction of CD18-independent migration. This is provoked by the finding that the macrophage-rich lung has been demonstrated to exhibit neutrophil influx that is CD18-independent or only partially dependent migration to a wide variety of stimuli, including hydrochloric acid, Strep. pneumoniae, LPS, C5a and IL-1α, whereas only PMA induced neutrophil migration seems to be fully CD18-dependent at this site (Doerschuk et al, 1990, and Hellewell et al, 1994). By contrast, both these studies showed similar stimuli produced neutrophil accumulation in sites supplied by the systemic circulation (which contained fewer macrophages) that was completely CD18-dependent.

This suggestion has been supported by several lines of evidence. Firstly, Strep. pneumoniae induced accumulation of neutrophils in the peritoneum is normally CD18-dependent, but can be induced to be CD18-independent by prior induction of peritoneal

macrophages by protease peptone injection or transplantation of macrophages from inflamed peritoneum of other animals (Mileski et al, 1990).

Secondly, in Winn and Harlan's study of the neutrophil influx into the peritoneum discussed above, administration of antibody against VLA-4 in addition to anti-CD18 antibody prevented the development of CD18-independence of neutrophil accumulation at 24 hours. This antibody does not bind to neutrophils, but binds to monocytes and in conjunction with anti-CD18 blocks their accumulation at inflammatory sites. Thus, the inhibition of late, CD18-independent neutrophil accumulation after administration of anti-VLA-4 is suggested to be secondary to the inhibition of monocyte recruitment.

Thirdly, Pseudomonas aeruginosa produces an acute pneumonia in which neutrophil influx is CD18-dependent. Recurrent pneumonia after one week, induced in the same portion of the lung, and produced at a time of increased alveolar macrophage numbers there, produces neutrophil accumulation that is CD18-independent (Kumasaka et al, 1996).

It does seem therefore that the macrophage is important in the induction of CD18-independent mechanisms of neutrophil accumulation when this occurs.

In the current study, neutrophil influx was largely (but not completely) CD18-dependent at 6 hours, and this remained equally true at 30 hours. There was no evidence that modulation of the use of β_2 integrins occurs during the course of pneuE.

3.6 Summary

1) The anti-CD18 antibody, 6.5e, was used to investigate the CD18 dependency of neutrophil influx early (6 hours) and late (30 hours) after the induction of E. coli pneumonia.

2) It was confirmed that 6.5e shows specific binding to the neutrophil surface *in vitro.*

3) Intravenous injection of 6.5e at an initial dose of 1 mg/kg followed by further doses of 0.5 mg/kg after 6 and 12 hours produced complete saturation of neutrophil surface CD18. Total CD18 expression was decreased.

4) This regime of 6 5e administration produced no change in neutrophil half-life or pattern of distribution in control rabbits.

5) Neutrophil influx to pneuE was inhibited by 6.5e in 6 and 30 hour groups as assessed by tissue gamma well counting and external scintigraphy. The inhibition was incomplete, implying a contribution of CD18-independent mechanisms. The degree of CD18-dependence was unchanged in the 30 hour compared with the 6 hour group. There was no evidence to support the hypothesis that control of the degree of utilisation of CD18-dependent and independent pathways during evolution of inflammation is a significant way in which the progression of the inflammatory process is controlled.

6) The apparent difference between this and the progressive CD18 independence of neutrophil influx in the peritoneum in response to E. coli and protease peptone (Winn, R. K. and Harlan, J. M., 1993) may be due to the different sites studied. Other reasons also exist, including the possibility that the presence of anti-CD18 antibody throughout the inflammatory response (as in the study of Winn and Harlan) itself induces the development of CD18-independent means of neutrophil recruitment.

4. THE EFFECT OF ANTI-RABBIT IL-8 ANTIBODY ON NEUTROPHIL INFLUX

4.1 Introduction

In vitro, neutrophils show the capacity for directed movement in response to a chemical gradient. This is known as chemotaxis (Harris, 1954, Caterina and Devreotes, 1991, and Zigmond, 1989).

As reviewed in the general introduction to this thesis, a variety of chemotactic substances have been known for some time, including LTB_4, C5a and bacterial products such as FMLP. These factors are not only chemotactic for the neutrophil but also for monocytes. The initial phase of inflammation is characterised by selective neutrophil accumulation, and this specificity was hard to account for on the basis of action of these 'traditional' chemotaxins. This has led to great interest in the more recently characterised chemokines. This family of mediators is split depending on characteristic structural motifs into the C-X-C chemokines (which include IL-8) and the C-C (archetypally MCP-1). The former characteristically cause selective neutrophil (and, to a lesser degree, lymphocyte) chemotaxis, whilst the latter cause selective monocyte chemotaxis (Leonard and Yoshimura, 1990).

It has long been presumed that once the neutrophil is immobilised by adhesion to vascular endothelium at the site of inflammation, it is induced to migrate into the tissues by the establishment of a chemotactic gradient (Harris, 1954). It has more recently been pointed out that stable chemical gradients may be difficult to achieve in the presence of fluid fluxes which may occur *in vivo*, and that chemotactic molecules may not be free, but fixed and presented on the surface of cells or on extracellular matrix in the process of haptotaxis (Rot,

1996 and Smith, 1992b), nevertheless the concept of chemotaxis retains some overall validity.

As well as inducing neutrophil chemotaxis, IL-8 has a variety of neutrophil activating activities, including stimulation of reactive oxygen intermediary generation, degranulation and phagocytosis. However, in addition to these proinflammatory activities, it also inhibits neutrophil migration across activated endothelium, and may in some circumstances inhibit influx to inflammatory sites *in vivo* (see general introduction for references). Clearly its rôle and importance *in vivo* need to be carefully examined.

IL-8 levels have been found to be elevated at a wide variety of inflammatory sites, including the lung, and in some cases this has been correlated with the number of neutrophils present. Functional interpretation of this finding is complicated by the fact that neutrophils may in themselves generate significant amounts of IL-8, so elevated IL-8 levels may be the result not the cause of increased neutrophil numbers (Bazzoni et al, 1991). Nevertheless, data has been produced to show anti-IL-8 antibody blocks *in vitro* chemotactic activity of biological fluids obtained from inflamed lungs, and that anti-IL-8 antibody may inhibit neutrophil influx into the lung (referenced in the general introduction).

A number of studies have been performed relating specifically to pneumonia. Chemotactic activity has been assessed in broncho-alveolar lavage fluid from patients with pneumonia (Hopkins et al, 1989). Three different chemotactic factors were identified; LTB_4, C5a and a factor with a molecular weight of around 10 kDa. The latter was presumably IL-8, but this had not been well characterised at the time. These samples were taken from patients with pneumonia at various stages of evolution. This is certainly interesting, but it is difficult to be certain how accurately such *in vitro* tests relate to *in vivo* functions. Bound molecules acting to promote haptotaxis rather than chemotaxis might be grossly under-represented in samples from inflamed sites, so that the importance of soluble chemotaxins in promoting directed movement *in vivo* might be grossly over-represented.

Increased IL-8 levels have been found in broncho-alveolar lavage fluid from patients with CAP (Boutten, 1996) and pneumonia requiring ventilatory support in the intensive care unit (Chollet-Martin, 1993). The latter study, but not the former, demonstrated elevated levels of plasma IL-8, the difference perhaps being accounted for by the difference in severity in the patients studied.

A further study of IL-8 level in broncho-alveolar lavage fluid demonstrated an elevation during pneumonia (Villard et al, 1995). They also showed that levels were similar to those found in ARDS and Pneumocystis pneumonia, but neutrophil levels were much the highest in bacterial pneumonia. They also demonstrated a correlation between IL-8 level and neutrophil numbers in ARDS and Pneumocystis pneumonia, but not in bacterial pneumonia. This indicates that there is no simple universal relationship between IL-8 and neutrophil presence, which in turn implies the function of IL-8 (and particular its importance compared to other chemotaxins) varies in different situations.

These studies suggest there might be a rôle for IL-8 in bacterial pneumonia. However, as they are all clinical studies, the timing of onset of disease is very difficult to define. In addition, patients were studied at different times during the evolution of their disease, and it is difficult to assess the relative contribution of IL-8 at different times in the inflammatory process.

Anti-IL-8 antibodies have been found to inhibit neutrophil influx when given before or just after acid instillation into the lung in rabbits (Folkesson, H. G., Matthay, M. A., Hébert, C. A., and Broaddus, V. C., 1995). No such experiments have been performed in bacterial pneumonia. In addtion, there is very little data examining whether the relative functional importance of IL-8 may change during the evolution of the inflammatory process.

A study was therefore performed to assess the effect of anti-IL-8 antibody on specific neutrophil influx in pneuS or pneuE, at 6 or 30 hours. The experimental procedure was analogous to that described in chapter three in which the effect of anti-CD18 antibody on

neutrophil migration was assessed. The inflammatory process was allowed to develop unmodified for 6 or 30 hours as appropriate. Anti-IL-8 antibody was then administered prior to the administration of ^{111}indium-labelled neutrophils. Specific influx of these cells to the pneumonic lung was then assessed as in previous experiments, with correction for blood pool effects by the use of autologous ^{99m}technetium-erythrocytes.

4.2 Methods

4.2.1 Studies with anti-IL-8 antibody

Rabbit recombinant IL-8 and mouse monoclonal anti-rabbit IL-8 antibody were provided under agreement with Genentech, Inc., San Francisco, USA. The antibody, ARIL.8.2, produced from clone 6G4.2.5, binds rabbit IL-8 with high affinity and cross reacts with human IL-8, but shows no affinity for other human chemokines (gro, platelet factor-4, β-thromboglobulin), other human cytokines (TNF-α, IL-1β) or other chemotactic factors (FMLP and C5a) (Broaddus et al, 1994). (Unfortunately, it is not possible to test against other rabbit chemokines, because these have mostly yet to be isolated). It is a whole IgG2a antibody. It was supplied at a concentration of 5.6 mg/ml in PBS and contained 0.12 eu/ml of endotoxin. IL-8 was supplied at 1.7 mg/ml in PBS, with an endotoxin content of 1.31 eu/ml.

4.2.1.1 In vitro *action of ARIL.8.2*

The *in vitro* activity was then checked by flow cytometrically assessing its ability to antagonise IL-8-induced neutrophil shape change. Neutrophils were prepared as described above. 100 μl aliquots of 10^8 per ml in Hanks Buffered Salt Solution with cations, with 10 mM MOPS (morpholinopropanesulphonic acid) were added to polystyrene test tubes (LP2 tubes, Merck, Lutterworth). Eight samples each were prepared with PBS control, and with IL-8 added to a final concentration of 10^{-9}, 10^{-8}, and 10^{-7} M. PBS control, and ARIL.8.2 to

final dilutions of 1:50, 1:25 and 1:10 were added to duplicates from each set of eight. The final volume was made up to 200 µl with PBS.

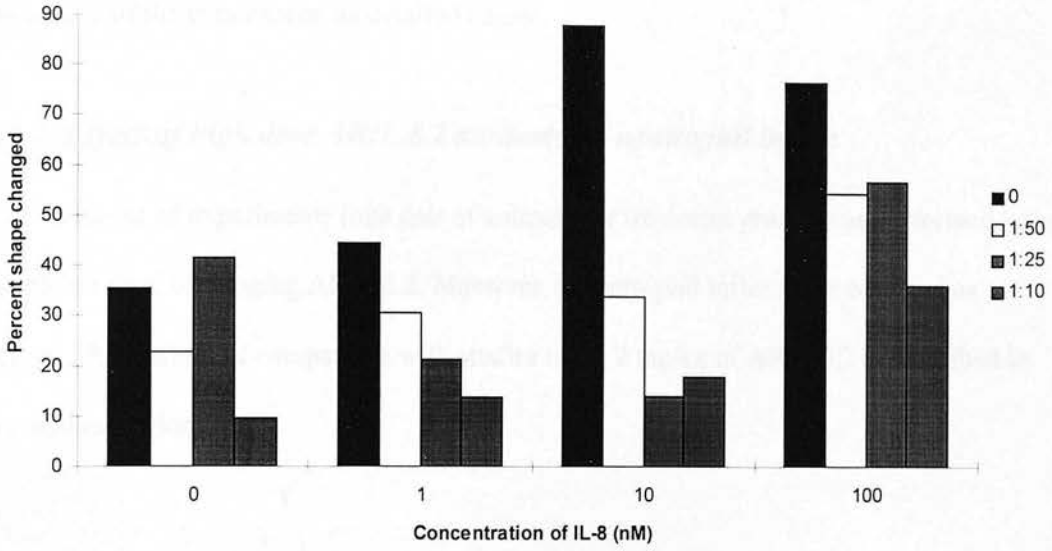
The tubes were incubated at 37° C with 5 % CO_2 for 30 minutes, following which they were fixed by the direct addition of 12 µl of 40 % (v/v) formaldehyde.

Neutrophils were then examined for shape change flow cytometrically. A plot of forward scatter against log side scatter produced a well demarcated population of non-shape changed neutrophils with low forward and log side scatter characteristics. This was gated on in the plots for control neutrophils. Shape changed neutrophils produced an increase in forward scatter. Neutrophils showing forward scatter above the control gate were expressed as percentage of total neutrophils to give the percentage of shape-changed neutrophils.

Thirty-five per cent of neutrophils were shape-changed at rest. In the absence of antibody, maximum shape change (88.2 %) was produced by 10^{-8} M IL-8 (see figure Figure 4-1). This could be returned to at least as low as control levels with a 1:50 dilution of ARIL.8.2. Although 10^{-7} M IL-8 produced slightly less shape change (77.1 %) this required 1:10 a dilution of antibody in order to return shape change to control values. Higher dilutions were only partially effective.

Figure 4-1 *In vitro* action of ARIL.8.2

The graph shows the effect of anti-IL-8 antibody on IL-8 induced neutrophil shape change. The x-axis shows concentration of IL-8, with graph series showing decreasing dilutions of anti-IL8 antibody. Data shows mean per cent shape change, assessed flow cytometrically, each data point being the mean of duplicate samples.



A further and less expected finding was that there was a tendency at all except the highest dose of IL-8 used for increased concentrations of antibody to produce shape change less than control values. Even control cells could have their shape change reduced by addition of antibody. This may represent autocrine or paracrine effects of IL-8 produced by the neutrophils.

4.2.1.2 Overall experimental plan for experiments examining the effect of anti-IL-8 antibody

The overall experimental plan for experiments to assess the effect of ARIL.8.2 on neutrophil influx was very similar to the experiments using anti-CD18 antibody (see Figure 4-2). 6 and 30 hour groups were studied, for pneuS and pneuE. 5 minutes prior to injection of ^{111}indium-labelled neutrophils, one of a pair of animals received 2 mg/kg ARIL.8.2, the other PBS as control. Antibody treated and PBS control pairs received In-wbc and bacterial inoculates from the same pools.

Indices of neutrophil influx were calculated as before, with differences between antibody and PBS control treated animals being evaluated by paired t-tests.

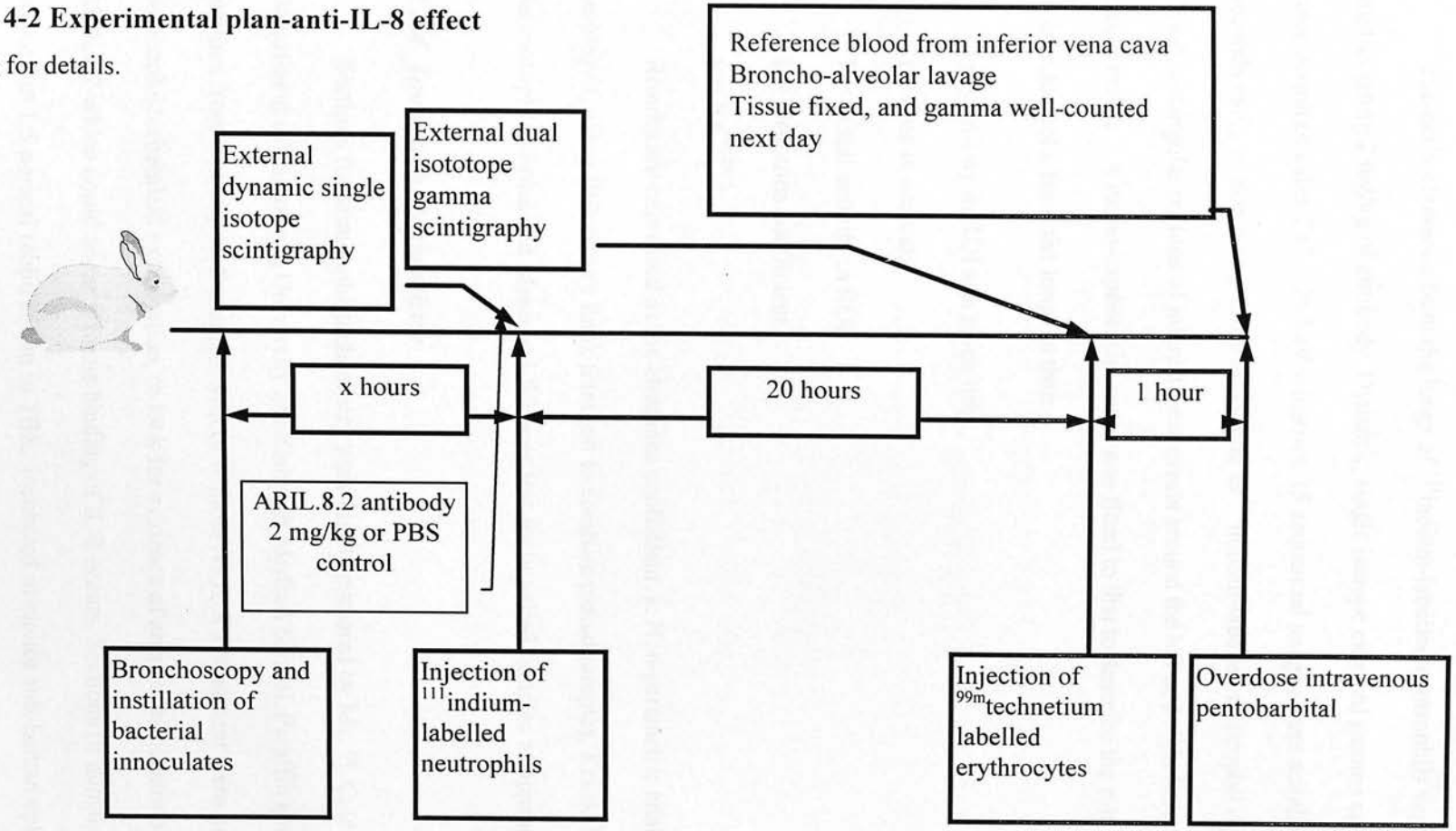
In addition, further gamma camera images and blood samples were obtained during the course of the experiment, as detailed below.

4.2.2 Effect of high dose ARIL.8.2 antibody on neutrophil influx

One set of experiments (one pair of animals per treatment group) was performed using the higher dose of 6 mg/kg ARIL.8.2. Measures of neutrophil influx were obtained as before. The method of comparison with studies using 2 mg/kg of ARIL.8.2 is described in the results section.

Figure 4-2 Experimental plan-anti-IL-8 effect

See text for details.



4.2.3 Neutrophil clearance experiments

The early clearance from the lungs of ^{111}indium-labelled neutrophils was examined in studies using 2 mg/kg of antibody. Dynamic, single isotope external gamma camera images were acquired using 247 $\pm$ 20 keV windows. 15 sequential images were acquired, over 20 seconds each, commencing at the same time as ^{111}indium-labelled neutrophil injection. Equal rectangular regions of interest were create around the left and right lung using the fourth image. A mono-exponential curve was fitted to this to describe the rate of clearance of labelled cells from the lung, so that:

y = activity in ROI at a given time

t = time in seconds

b = initial activity in ROI

a = clearance coefficient

$y = b.e^{-at/20}$

Results are expressed as the clearance coefficient, a. Non-parametric analysis is employed, using Wilcoxon's Rank Sum test to compare paired samples, Kruskall-Wallis test for multiple samples and Mann and Whitney test for unpaired samples as appropriate.

4.2.4 Immunohistochemistry

Sections for immunohistochemistry were kindly prepared by Ms. H. Caldwell, Department of Pathology, University of Edinburgh Medical School. Paraffin embedded sections from 6 hour pneuS animals with or without ARIL.8.2 treatment were used for immunohistochemical examination, to look for evidence of areas of concentrated binding of ARIL.8.2 which could occur if tissue binding of IL-8 occurs. Sections of human lung were exposed to 1:5 normal rabbit serum in TBS, incubated in mouse anti-human epithelial

membrane antigen (Dako, High Wycombe) and (after washing in TBS) used as positive controls.

Sections were then incubated with normal rabbit serum and washed twice. They were incubated in biotinylated goat anti-mouse antibody (Sigma, Poole) diluted 1:200 in TBS for 30 minutes. After washing in TBS, they were incubated in avidin/bioitn-HRP for 30 minutes as per the suppliers instructions (Dako), and re-washed. Endogenous peroxidase was blocked with glucose oxidase, using 20 minutes incubation in a solution containing β-D glucose 100mM, glucose oxidase 100 U/ml, sodium azide 10mM and 100 mM PBS. After a further wash, sections were incubated with diaminobenzidine substrate solution, 0.5 % (w/v). After rinsing in tap water, sections were counterstained with 0.1 % Methyl Green. After dehydration, sections were mounted for examination.

4.2.5 Plasma and broncho-alveolar lavage IL-8 levels

From a random selection of pairs of animals treated with 2 mg/kg of ARIL.8.2 or PBS control (weighted to give even distribution across groups), 2 ml blood samples were taken into tubes containing EDTA immediately prior to administration of antibody, and immediately post mortem. These were placed on ice until centrifuged at 2500g for 10 minutes at 4°C. Plasma was aspirated, ensuring no erythrocytes were removed, and stored in 1.5 ml Eppendorf tubes at -70°C until assay for IL-8 content.

Broncho-alveolar lavage fluid from each animals in this experiment was centrifuged at 2500g for 6 minutes at 4°C. Aliquots of 1.5 ml of supernatant were stored in Eppendorf tubes at -70°C until assay for IL-8.

IL-8 assays were kindly performed by Genentech, Inc., under the direction of Dr. C. Hébert, using standard ELISA procedures described previously (Folkesson et al,1995).

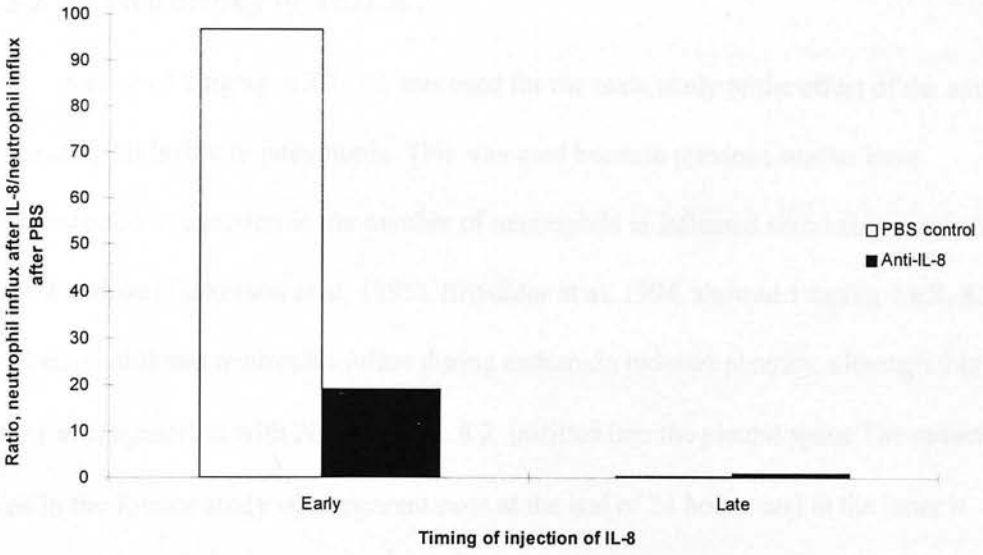
4.2.6 In vivo *studies of neutrophil influx into the skin*

In four pairs of animals in the experiment using 2 mg/kg of ARIL.8.2, the effect of ARIL.8.2 on neutrophil influx to the skin was examined. The dorsal skin of the rabbit was shaved. Immediately after administration of ARIL.8.2 or PBS control, 2 sites were injected intradermally with 3 x 10^{-9} moles of recombinant rabbit IL-8 in 100 µl of PBS without cations, and two sites were injected with 100 µl PBS without cations as control. This procedure was repeated just prior to the injection of ^{99m}technetium labelled erythrocytes. Thus, IL-8 was injected early (allowing 21 hours for In-wbc migration) or late (allowing 1 hour). Immediately post-mortem, 1 cm. diameter portions of skin were excised from the injection sites. ^{111}indium and ^{99m}technetium activities were measured by gamma well counting and corrected for weight of the sample. Calculations exactly analogous to those performed for the lung were carried out, to subtract the expected blood pool from total ^{111}indium activity, and thus measure the specific influx of labelled neutrophils into the skin. The mean of duplicate samples was determined. Results were expressed ratio of neutrophil influx in response to IL-8, and the neutrophil influx in response to PBS. The median of this value for the four experiments performed is quoted.

There was a large In-wbc influx in response to intradermal IL-8 given 21 hours *ante mortem* in non-antibody treated control animals, but there was no response to intradermal IL-8 given 1 hour *ante mortem* to either control or antibody treated animals. ARIL.8.2 markedly inhibited the 21 hour response.

Figure 4-3 Neutrophil influx into skin in response to intra-dermal IL-8

The graph show neutrophil influx into the skin, as assessed by tissue gamma well counting, in response to intra-dermal IL-8 (3×10^{-9} moles) or PBS control. Animals were treated with ARIL.8.2 or control. Duplicate assessments were performed in each rabbit and the mean determined. The result is expressed as the ratio of neutrophil influx in response to IL-8 and to PBS. The median of four such experiments is presented for each data point.



4.3 Discussion of methods

4.3.1 In vitro *activity of ARIL.8.2*

Chemotaxins cause neutrophils to change shape. The ability of anti-IL-8 to antagonise IL-8 induced neutrophil shape change was used to prove that the antibody had IL-8 blocking activity *in vitro* as supplied. Shape change was assessed flow cytometrically, a method found to give close correlation with manual counts (Cole et al, 1995), and to correlate well with chemotaxis as assessed by Boyden chambers.

In the experiment performed there was a relatively high level of basal neutrophil shape change, indicating some initial cell activation. Addition of IL-8 caused additional shape change as expected, with an optimum dose of 10^{-8} M. Antibody ARIL.8.2 dose dependently inhibited neutrophil shape change. In fact, higher concentrations of ARIL.8.2 sometimes decreased shape change beyond control levels. As noted the neutrophils exhibited some basal activation. It is possible they were themselves producing IL-8, and in

control cells this was acting by autocrine or paracrine pathways to produce shape change. Inhibition of this would reduce control shape change. Overall, it is clear ARIL.8.2 dose dependently inhibits the action of IL-8 *in vitro.*

4.3.2 In vivo *activity of ARIL.8.2*

A dose of 2mg/kg ARIL.8.2 was used for the main study of the effect of the antibody on neutrophil influx in pneumonia. This was used because previous studies have demonstrated a reduction in the number of neutrophils in inflamed sites using this antibody at such a dose (Folkesson et al, 1995). Broaddus et al, 1994, showed 1 mg/kg ARIL.8.2 markedly inhibited neutrophil influx during endotoxin induced pleurisy, although this was given in conjunction with 200 μg ARIL.8.2 instilled into the pleural space The reduction seen in the former study was apparent even at the end of 24 hours, and in the latter it continued to the end of the study, at 6 hours.

Intradermal IL-8 caused neutrophil influx into the skin when injected early (allowing 21 hours for migration) but not when injected late (allowing 1 hour for migration). Similar results have been found with commensurate IL-8 levels in sheep, dogs, mice and man (Seow et al, 1994, Zwahlen et al, 1994, Zhang et al, 1995, and Hechtman et al, 1991, Leonard et al,1991, respectively). In these studies, the earliest time at which neutrophil accumulation was detected was 30 minutes. The lack of accumulation seen after 1 hour in the current study is likely to be due to the relative lack of sensitivity of the technique used, compared to histological scoring or total tissue myeloperoxidase used in the other studies.

Antibody ARIL.8.2 was able to inhibit neutrophil influx into the skin in response to intradermal IL-8. This confirms that, as expected, it is able to antagonise IL-8 induced neutrophil influx, as assessed by accumulation of In-wbc, *in vivo*.

The main experimental plan used in this chapter is very similar to that used in chapter 3 to assess the effect of the anti-CD18 antibody, 6.5e, and similar general considerations

apply. The methods are further discussed in the main discussions section for this chapter, in the light of the results obtained.

4.4 Results

4.4.1 Effect of standard dose ARIL.8.2 on neutrophil influx into lung during pneumonia

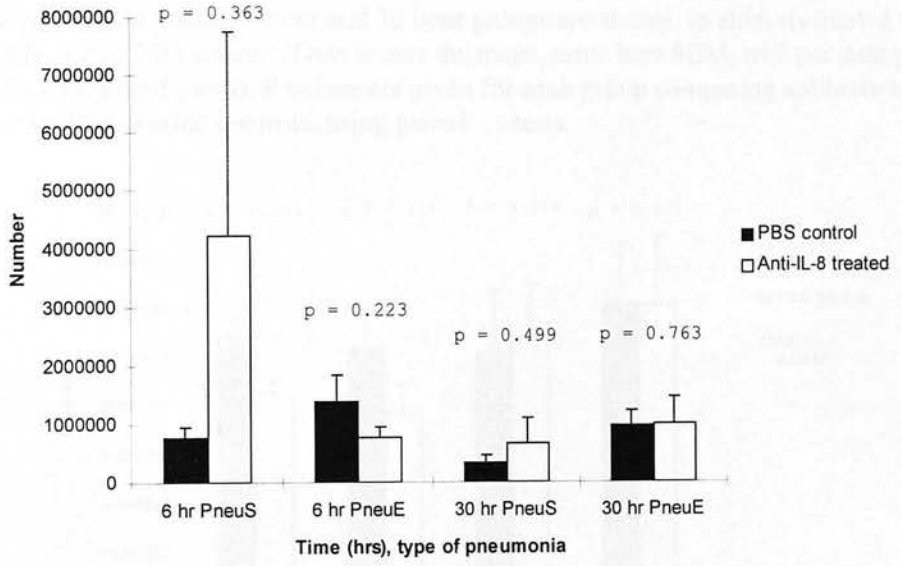
One untreated animal from the 30 hour E. coli group died during the study, so this and its paired animal were excluded from analysis.

4.4.1.1 Effect of ARIL.8.2 on total neutrophil recovery from broncho-alveolar lavage

There was no significant difference in total neutrophil recovery from broncho-alveolar lavage fluid in either type of pneumonia at either time point. Overall no trend to enhancement or inhibition of neutrophil recovery was apparent.

Figure 4-4 Effect of ARIL.8.2 on neutrophil recovery from broncho-alveolar lavage

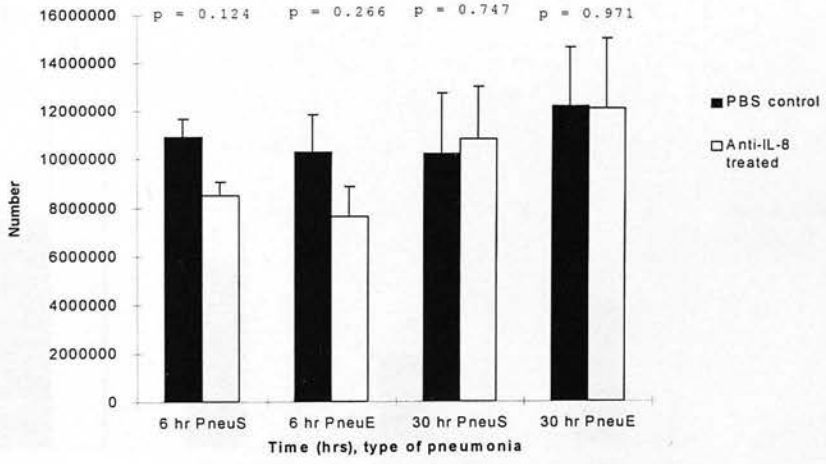
The graph shows the total recovery of neutrophils from total broncho-alveolar lavage in pneuS and pneuE. 6 hour and 30 hour groups are shown, in animals treated with either ARIL.8.2 or PBS control. Data shows the mean, error bars SEM, n=7 per data point (except 30 hour pneuE, n=6). P values are given for each group comparing antibody treated animals with PBS treated controls, using paired t tests.



4.4.1.2 Effect of ARIL.8.2 an total alveolar macrophage recovery from broncho-alveolar lavage.

Figure 4-5 Effect of ARIL.8.2 on alveolar macrophage recovery from broncho-alveolar lavage

The graph shows the total recovery of alveolar macrophages from total broncho-alveolar lavage in pneuS and pneuE. 6 hour and 30 hour groups are shown, in animals treated with either ARIL.8.2 or PBS control. Data shows the mean, error bars SEM, n=7 per data point (except 30 hour pneuE, n=6). P values are given for each group comparing antibody treated animals with PBS treated controls, using paired t-tests.

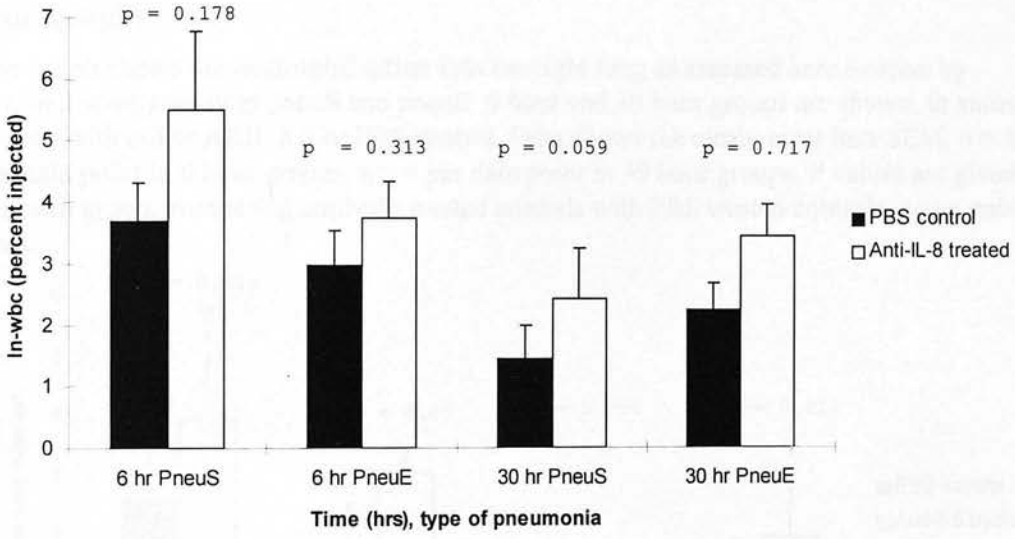


The administration of ARIL.8.2 had no significant effect on the number of alveolar macrophages recovered from broncho-alveolar lavage in either type of pneumonia, in either time group. There was no overall trend to either increased or decreased numbers apparent.

4.4.1.3 Effect of ARIL.8.2 on neutrophil influx as assessed by tissue gamma well counting.

Figure 4-6 Effect of ARIL.8.2 on neutrophil influx as assessed by tissue gamma well counting

The graph shows the neutrophil influx into the right lung as assessed *post mortem* by tissue gamma well counting in pneuS and pneuE. 6 hour and 30 hour groups are shown, in animals treated with either ARIL.8.2 or PBS control. Data shows the mean, error bars SEM, n=7 per data point (except 30 hour pneuE, n=6). P values are given for each group, comparing antibody treated-animals with PBS treated controls, using paired t tests.



ARIL.8.2 produced no significant effect on In-wbc into pneuS or pneuE, as assessed by tissue gamma well counting. However, in both time groups in pneuS and pneuE, the In-wbc influx was greater in antibody-treated than in PBS control animals.

Historical data from unbronchoscoped controls was used to calculate mean change in influx for this and other data categories as follows:

Overall change of neutrophil influx (per cent) $= \Delta_{inf}$
Neutrophil influx after ARIL.8.2 $= PMN_{\alpha IL\text{-}8}$
Neutrophil influx after control treatment $= PMN_{PBS}$
Neutrophil influx in unbronchoscoped controls $= PMN_{cont}$

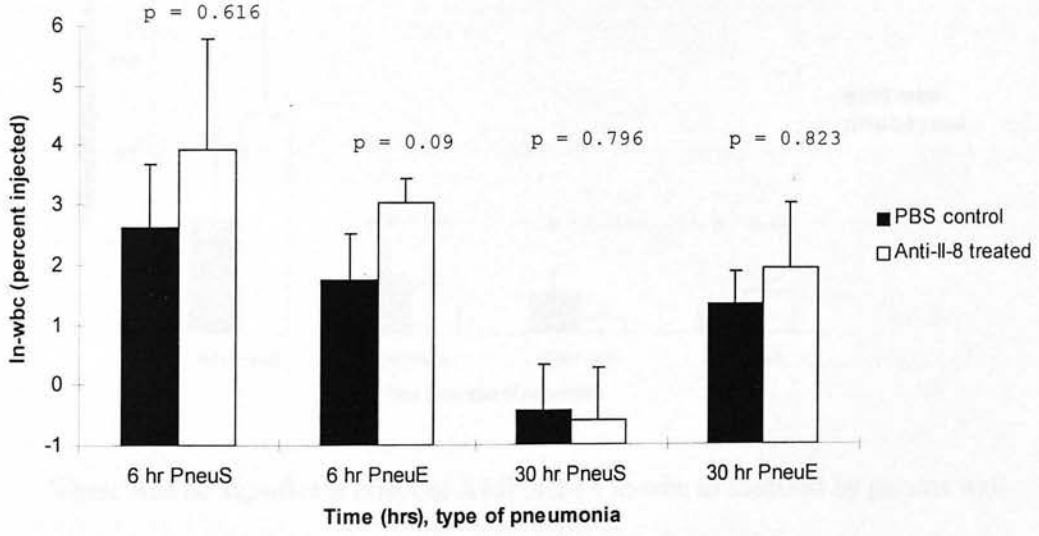
$$\Delta_{inf} = \left(\frac{[PMN_{\alpha IL\text{-}8} - PMN_{cont}]}{[PMN_{PBS} - PMN_{cont}]} \right) \times 100$$

The changes of mean neutrophil influx after ARIL.8.2 treatment in 6 and 30 hour groups in pneuS were 153.0 %, 183.3 % respectively, and in 6 and 30 hour groups in pneuE were 128.9 % and 160.3 % respectively.

4.4.1.4 Effect of ARIL.8.2 on neutrophil influx as assessed by external gamma scintigraphy

Figure 4-7 Effect of ARIL.8.2 on neutrophil influx as assessed by external scintigraphy

The graph shows the neutrophil influx into the right lung as assessed *ante mortem* by external scintigraphy in pneuS and pneuE. 6 hour and 30 hour groups are shown, in animals treated with either ARIL.8.2 or PBS control. Data shows the mean, error bars SEM, n = 5 per data point in 6 hour groups, n = 4 per data point in 30 hour groups. P values are given for each group, comparing antibody treated animals with PBS treated controls, using paired t tests.



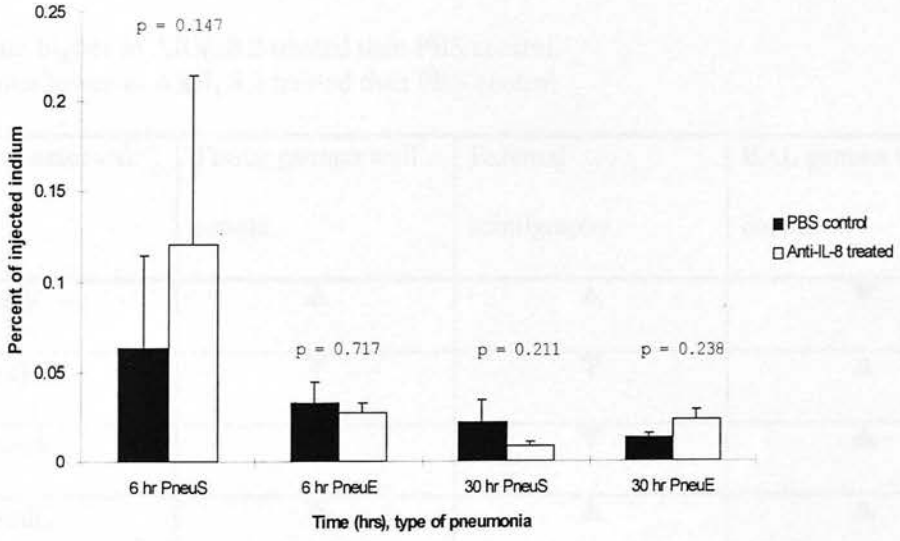
Administration of ARIL.8.2 produced no significant affect on neutrophil influx as assessed by external gamma scintigraphy. However, in three out of 4 groups there was a trend for increased In-wbc influx after treatment, the only exception being the 30 hour pneuS group. The augmentation of mean In-wbc influx calculated as for the data obtained with tissue gamma well counts was 130.1 %, 86.6%, 138.0 % and 120.7 % for 6 and 30 hour

pneuS and 6 and 30 hour pneuE groups respectively. (Collection of data on all 7 sets of experiments was precluded by gamma camera malfunction).

4.4.1.5 Effect of ARIL.8.2 on broncho-alveolar lavage neutrophil influx as assessed by BAL gamma well counting

Figure 4-8 Effect of ARIL.8.2 on broncho-alveolar lavage neutrophil influx as assessed by tissue gamma well counting

The graph shows the neutrophil influx into the right lung as assessed post mortem by tissue gamma well counting of broncho-alveolar lavage in pneuS and pneuE. 6 hour and 30 hour groups are shown, in animals treated with either ARIL.8.2 or PBS control. Data shows the mean, error bars SEM, n=7 per data point (except 30 hour pneuE, n=6). P values are given for each group, comparing antibody treated animals with PBS treated controls, using paired t tests.



There was no significant effect of ARIL.8.2 on In-wbc as assessed by gamma well counts in any group. Overall, there was no trend either to inhibition or augmentation of In-wbc influx.

4.4.2 Effect of high dose anti-IL-8 on neutrophil influx into lung during pneumonia.

The standard dose of 2 mg/kg ARIL.8.2 used in the initial experiments produced no inhibition of neutrophil influx as assessed by any of the methods used. Further studies were

undertaken to examine whether a higher dose of ARIL.8.2 might produced measurable inhibition of neutrophil influx. Only one set of experiments was undertaken for practical purposes; the experiments required very large amounts of antibody. An antibody-treated and PBS control pair of animals was examined at each time point for both types of pneumonia, with three measures of neutrophil influx obtained in each animal as with the low dose studies. This gave 12 paired measures of neutrophil influx. Influx was greater in the treated animal than its PBS treated control pair in 7 measures, and was less in 5.

Table 4-1 The effect of high dose ARIL.8.2 on neutrophil influx in pneumonia

The table shows whether neutrophil influx measures were increased or decreased in high-dose (6mg/kg) anti-IL-8 antibody treated rabbits than in PBS treated controls, in pneuS and pneuE, in 6 and 30 hour groups.

▲ Value higher in ARIL.8.2 treated than PBS control
▼ Value lower in ARIL.8.2 treated than PBS control

In-wbc as assessed by;	Tissue gamma well counts	External scintigraphy	BAL gamma well counts
6 hr pneuS	▲	▲	▼
6 hr pneuE	▼	▼	▲
30 hr pneuS	▲	▼	▲
30 hr pneuE	▼	▲	▲

To compare data of animals treated with high dose (6 mg/kg) ARIL.8.2 directly with data from the studies using standard dose (2 mg/kg) ARIL.8.2, the ratio of influx measurement in each treated animal and its matched control was examined. The ratio obtained with high dose treatment was compared with the interquartile range for such ratios obtained in the standard dose studies. The value obtained in the high dose study fell above the interquartile range for the standard dose study in 5 cases, within it in 4 cases, and below it in 3 cases.

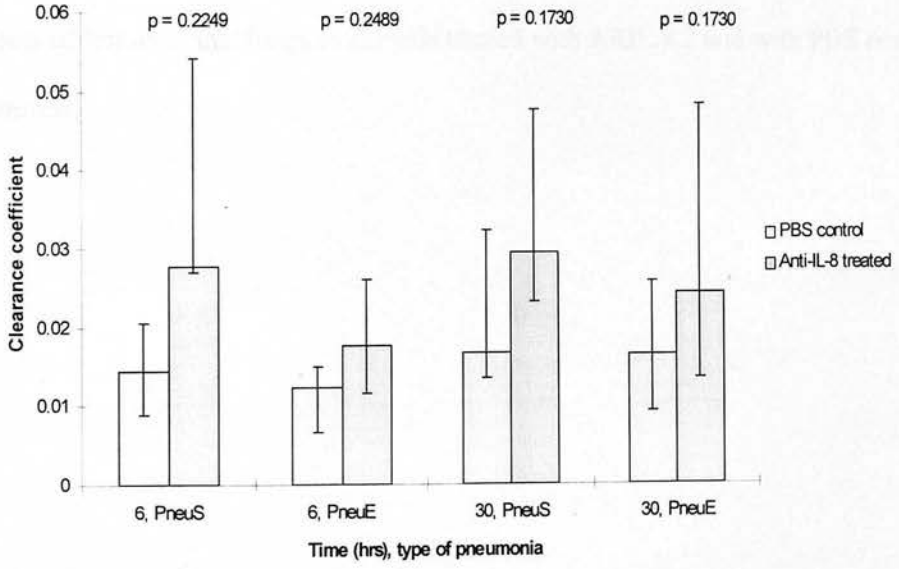
Overall, the data with the two doses of ARIL.8.2 were very similar. Even with the very high dose of 6 mg/kg ARIL.8.2, no trend to inhibition of neutrophil was seen.

Data for measures of neutrophil numbers in broncho-alveolar lavage fluid likewise failed to show any tendency to decrease with high dose ARIL.8.2 treatment.

4.4.3 Lung clearance coefficients for neutrophils

Figure 4-9 Neutrophil lung clearance coefficients

The graph shows the initial clearance coefficient of neutrophils from the right lung in pneuS and pneuE in 6 and 30 hour groups. Lung region of interest ^{111}indium activity was measured every 20 seconds from 1 to 5 minutes after injection of ^{111}indium-labelled neutrophils, and a monoexponential curve fitted to change in activity with time. The time constant is plotted for ARIL.8.2 treated or PBS control rabbits, for both pneuS and pneuE. Data shows medians and interquartile ranges, n = 5 to 7 per group.



In 24 animals equally distributed across groups, the clearance rate of In-wbc from left and right lungs was assessed by gamma scintigraphy. The activity over each lung field was measured each 20 seconds from 60 to 300 seconds after injection of In-wbc. A monoexponential curve was fitted. Good fits were obtained.

Table 4-2 Summary of monoexponential goodness of fit for clearance data.

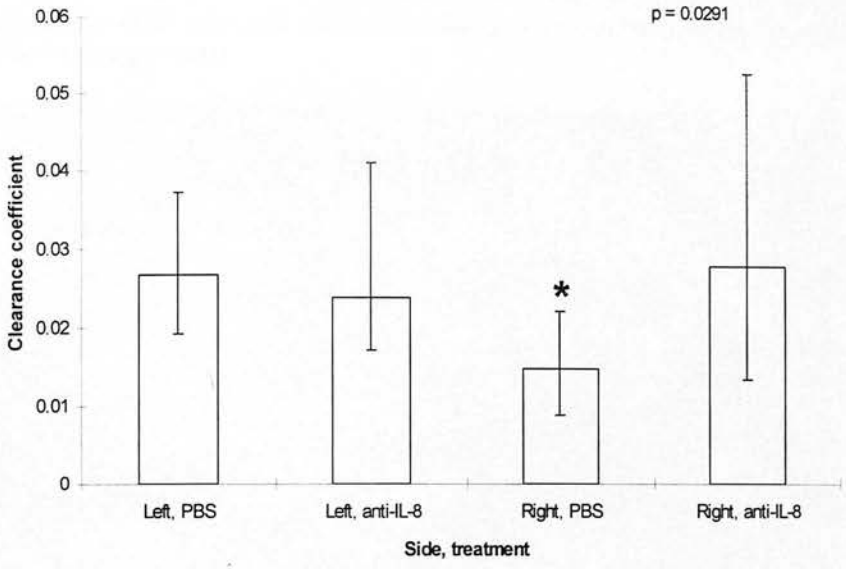
The table shows the medians and interquartile ranges for the residual squares, F statistics, and p values for monoexponential fits to neutrophil clearance from the lung (n = 24).

	Rsq	F	Significance
Lower quartile	**0.4195**	**7.265**	**<0.0001**
Median	**0.6035**	**15.205**	**0.003**
Upper quartile	**0.7847**	**38.012**	**0.0237**

At each time point in both types of pneumonia, the clearance coefficient for the right lung was higher in ARIL.8.2 treated than in untreated animals. This did not reach statistical significance in any individual group using Wilcoxon's Rank Sum test. This may be due to small numbers (n = 6 in each group) so the data was also pooled, and the clearance coefficients of left and right lungs in animals treated with ARIL.8.2 and with PBS control was examined.

Figure 4-10 Neutrophil lung clearance summary

The graph shows pooled data from pneuS and pneuE 6 and 30 hour groups. Data is presented for initial neutrophil clearance coefficient for the right and left lungs from animals treated with ARIL.8.2 and controls (as described in figure 3.10). The data is presented as medians and interquartile ranges, n = 24 per group. There was a significant difference between the groups (p = 0.029, Kruskall-Wallis test). * shows group differing from all others on *post hoc* testing with Mann and Whitney test ($p<0.05$); no significant differences were found between other groups.



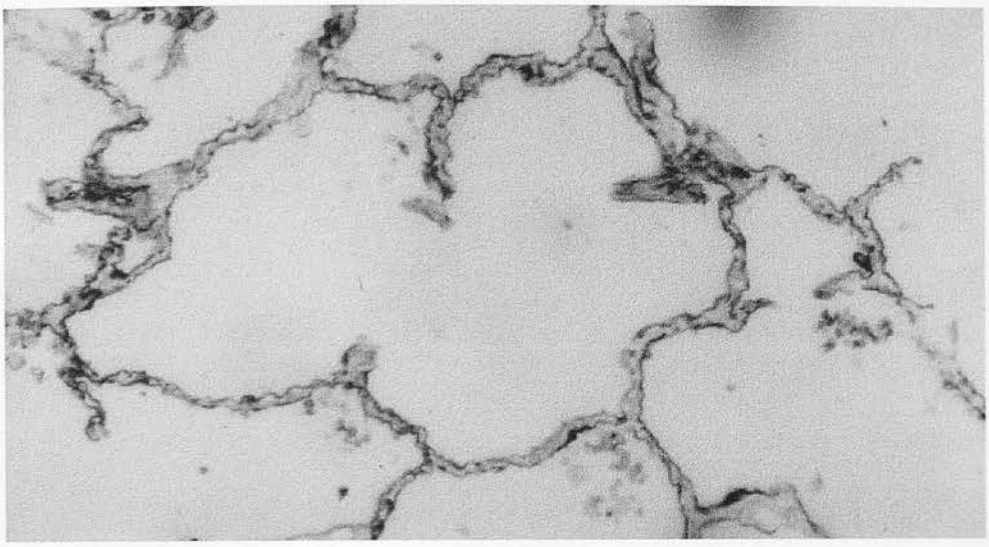
There was a significant difference between the clearance coefficient for In-wbc from the four groups (p = 0.0291 by Kruskal-Wallis test). Mann-Whitney test showed the clearance coefficient was significantly lower in the right lung of PBS control animals than the right lung of ARIL.8.2 treated animals, and the left lung of either animals. This shows that In-wbc were cleared more slowly from lungs with pneumonia than normal lungs, and this abnormality could be corrected by administration of ARIL.8.2.

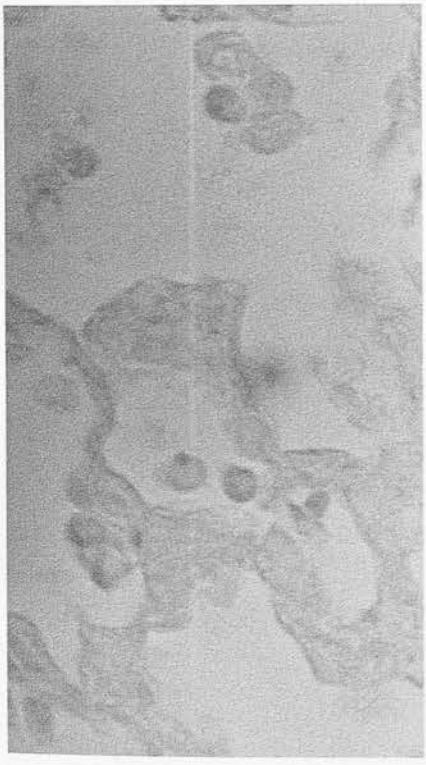
4.4.4 Immunohistochemistry of pneumonia

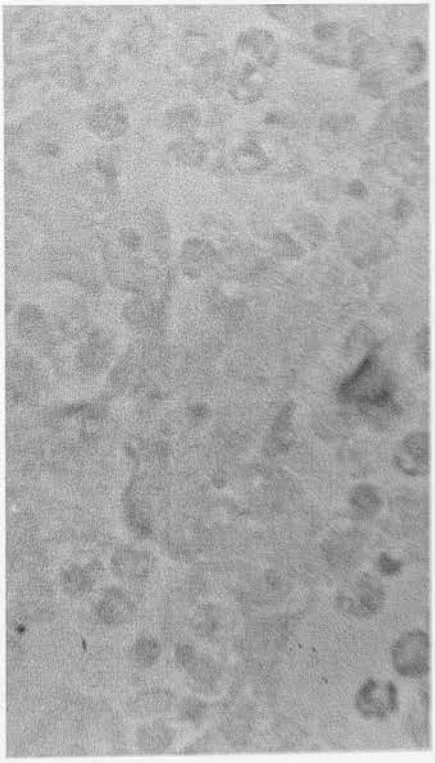
Binding of IL-8 to vascular endothelium has been observed. If this occurs during pneumonia, it is possible that the ARIL.8.2 binds here secondarily. Immunohistochemical staining of the lung was therefore performed, using sheep anti-mouse antibody to detect any localised ARIL.8.2. No evidence of such tissue localisation could be detected.

Figure 4-11 Immunohistochemistry of pneumonia

The photomicrographs shown were taken at an om of x400. The top photomicrograph shows normal human lung stained with mouse anti-EMA as a positive control for the biotinylated goat anti-mouse IgG second antibody. Clear staining of the epithelial membrane is apparent.
The lower left photomicrograph shows lung from an animal in the 6 hour pneuE group that received intravenous ARIL.8.2, and the lower right photomicrograph shows its PBS treated control pair. No localised second antibody mediated staining is shown, demonstrating ARIL.8.2 is not localised to inflamed lung *in vivo*.





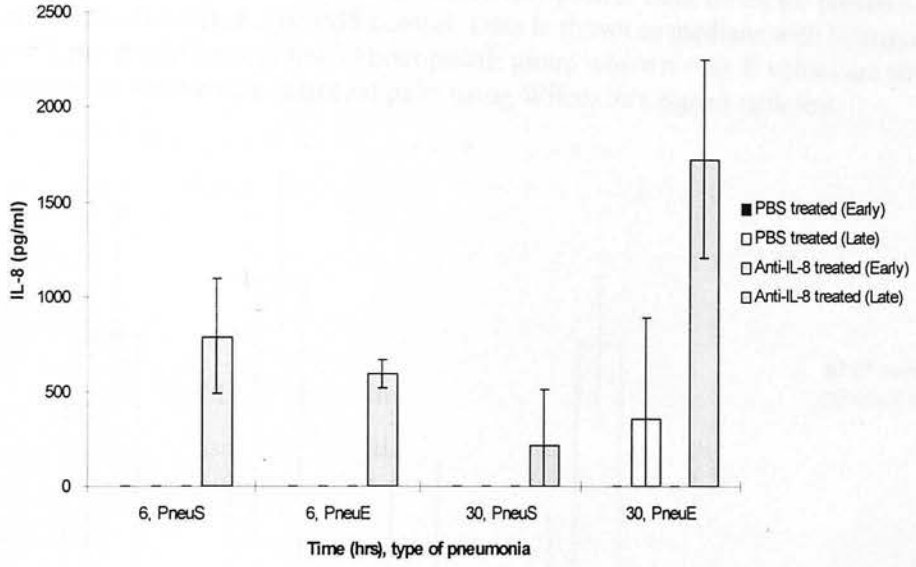


4.4.5 Plasma and broncho-alveolar lavage IL-8 levels.

4.4.5.1 Plasma IL-8 levels

Figure 4-12 Levels of IL-8 in plasma

The graph shows the plasma levels of IL-8 in pneuS and pneuE in 6 and 30 hour groups. Measurements were taken immediately before injection of ARIL.8.2 ("early") or immediately *post mortem* ("late"). Data is presented as medians and interquartile ranges. n = 3 to 5 per group.



Plasma IL-8 was measured immediately prior to administration of anti-IL-8 or PBS control (early) or immediately post mortem (late) in a random selection of treated and control pairs (n = 2 to 5 pairs per group). Because of the small number of samples, data was not subjected to formal statistical analysis. Early, IL-8 was below the limits of detection in both types of pneumonia in both 6 and 30 hour groups. IL-8 was detectable late in PBS controls only in some animals in the 30 hour pneuE group. By contrast, IL-8 was present at the late sampling point at high levels in nearly all animals after ARIL.8.2 treatment. This was true in all groups.

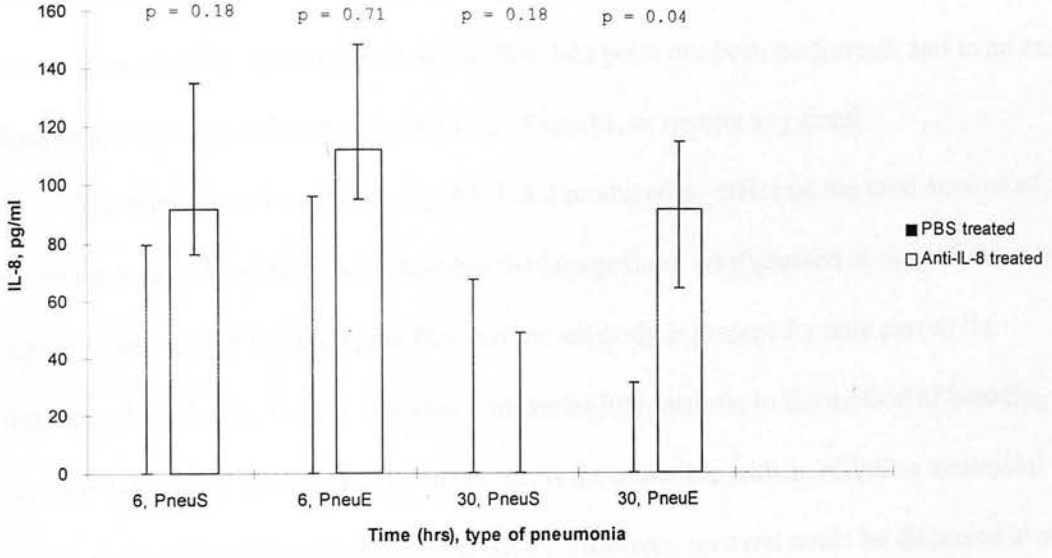
Overall, the data show that in both types of pneumonia at both time points, the administration of ARIL.8.2 was associated with an increase in plasma IL-8. Pooling all data, the early IL-8 was undetectable, but rose to a median of 653.4 pg/ml immediately *post*

mortem in animals receiving ARIL.8.2. In PBS controls, plasma IL-8 was also undetectable early, but no elevation was detected in samples taken immediately *post mortem* (median undetectable).

4.4.5.2 BAL IL-8 levels

Figure 4-13 Broncho-alveolar lavage IL-8 levels

The graphs show plasma IL-8 levels in broncho-alveolar lavage fluid from the right lung form 6 and 30 hour groups of animals with pneuS and pneuE. Data series are presented for animals treated with ARIL.8.2 or PBS control. Data is shown as medians with interquartile ranges, n = 7 per group (except the 30 hour pneuE group where n = 6). P values are shown for comparison of treated and untreated pairs using Wilcoxon's signed rank test.



Broncho-alveolar lavage IL-8 levels were measured in all animals. Median IL-8 was undetectable in PBS controls. Median IL-8 was elevated in 3 of the 4 study groups in ARIL.8.2 treated animals (the exception being the 30 hour pneuS group). This only reached significance in the 30 hour pneuE group.

Pooling the data, IL-8 was higher in bronchoalveolar lavage from ARIL.8.2 treated animals than in PBS control ($p = 0.01625$, $n = 26$, Wilcoxon's signed rank test).

4.5 Discussion

4.5.1 Effects of 2 mg/kg ARIL.8.2 on broncho-alveolar lavage cell numbers and neutrophil influx

In the data presented, it is clear there is an outlying data point in the 6 hour pneuS group. In one case there was a dramatically and unusually high influx of neutrophils that was reflected in all measures, including total neutrophil recovery from lavage. This was unusual not just for this study, but was also markedly different from values reported in chapters two or three. As it is not clear why this occurred, the data point has been retained in the primary analysis. Re-analysis without this data point has been performed, and in no case does this materially affect the significance of results, or reverse any trend.

The administration of 2 mg/kg ARIL.8.2 produced no effect on the total number of neutrophils recovered from broncho-alveolar lavage fluid. As discussed in chapter three with regard to anti-CD18 antibody, the fact that the antibody is present for only part of the duration of the inflammation, together with variability intrinsic to the method of broncho-alveolar lavage, do not necessarily mean this is incompatible with an effect on neutrophil influx, even if clearance rates remain constant. However, no trend could be discerned at all.

ARIL.8.2 produced no effect on the number of alveolar macrophages recovered from broncho-alveolar lavage fluid. In view of the fact that IL-8 has never been shown to be chemotactic for the monocyte/macrophage lineage, this is entirely expected.

More unexpected was the finding that ARIL.8.2 had no significant effect on the influx of neutrophils to the lung as assessed by tissue gamma well counting. Indeed, there was a clear trend at both time points, and in both types of pneumonia, for increased neutrophil influx. Similar non-significant trends were seen in 3 out of 4 groups when neutrophil influx was assessed by *ante mortem* gamma scintigraphy.

In view of these findings and the previously discussed technical limitations of the method, it was not surprising that neutrophil influx as assessed by gamma well counting of broncho-alveolar lavage fluid gave no statistically significant change. No trend was apparent either.

It has been noted earlier that the antibody used in this experiment causes reduced neutrophil accumulation to the pleura after instillation of LPS (Broaddus et al, 1994). It also inhibits neutrophil accumulation in the lung after hydrochloric acid instillation (Folkesson et al, 1995). In the latter, this was true even after 24 hours, using the same dose of antibody as in the current study. In the 3 kg rabbits, assuming a blood volume of 70 ml/kg, 2 mg/kg antibody would be diluted approximately 1:250. *In vitro,* 1:50 dilution of antibody was found to completely inhibit the action of as much as 10^{-8} M IL-8, the optimum dose for induction of neutrophil shape change. *In vivo,* the highest plasma level of IL-8 measured at any time was 10^{-10} M, and in all cases measured prior to ARIL.8.2 administration was less than 1.6×10^{-12} M, the lower limit of detection of the assay used for plasma estimations. Both previous experiments and extrapolation from *in vitro* results suggests that in the blood, ARIL.8.2 is present in sufficient amounts to block the IL-8 activity present.

It is much more difficult to calculate relevant tissue concentrations. Transfer of antibody into inflamed tissue as a consequence of increased vascular permeability is to be expected. Broncho-alveolar lavage IL-8 levels was rarely more, and often less, than 10^{-10} M. Although this represents a dilution of epithelial lining fluid, it seems likely antibody should be present in sufficient quantities to significantly inhibit IL-8 at least at close to sites of neutrophil emigration because of vascular leak in the capillaries at sites of inflammation.

If released IL-8 is tissue bound to a significant degree, its local activity might exceed that which is apparent by measurement of its concentration in biological fluids. The lack of localised binding of ARIL.8.2 at the site of inflammation demonstrated by immunohistochemistry makes this highly unlikely.

Nevertheless, it is important to ensure the lack of inhibition of neutrophil influx by ARIL.8.2 during pneumonia is not due to inadequate dosage. Therefore further experiments were performed with higher doses of antibody.

4.5.2 Effects of 6 mg/kg ARIL.8.2 on broncho-alveolar lavage cell numbers and neutrophil influx

Pairs of animals were treated with 6 mg/kg ARIL.8.2 or PBS control, a dose of antibody that should produce approximately 1:90 antibody dilution intravascularly. One pair each was studied for 6 and 30 hour groups in pneuS and pneuE. To facilitate comparison with low dose (2 mg/kg ARIL.8.2) studies, the ratio of neutrophil influx in the treated to the untreated animal was calculated, and compared to the interquartile ranges for similar values in the low dose study. Some values fell above, some below and some within the interquartile range, showing the high dose antibody produced similar results over all. Certainly no trend to inhibition of neutrophil influx could be discerned. There is thus no evidence that an inadequate dose of ARIL.8.2 explains its inability to inhibit the neutrophil influx.

4.5.3 Does the experiment measure neutrophil influx?

The validity of the basic experimental method has already been discussed in chapter two. ^{111}indium has been shown to remain predominantly cell bound during the course of the experiment. Injection of chelated ^{111}indium without incubation with cells results in its distribution throughout the blood pool, without accumulation at the inflammatory site as determined by gamma scintigraphy, whereas chelated ^{111}indium pre-incubated with neutrophil leads to its localisation at the inflammatory site. Autoradiography of the lung shows ^{111}indium within the tissues to be predominantly cell associated.

In addition, as discussed in chapter three, the anti-CD18 antibody 6.5e produced a readily detectable inhibition of neutrophil influx with a very similar experimental protocol. This was consistent with its known actions *in vitro* and in other *in vivo* experiments.

Therefore this model measures neutrophil influx, and is capable of detecting its modulation by specific interventions. It must be concluded that systemic inhibition of IL-8 is incapable of inhibiting neutrophil influx at 6 or 30 hours in pneuS or pneuE; indeed, it may actually enhance it.

It is notable that after gut ischaemia-reperfusion, preliminary data shows the anti-IL-8 antibody used in this study inhibits neutrophil influx to post-ischaemic gut, but not influx into the lung (C. Hébert, personal communication). Other studies (utilising different antibodies) have shown inhibition of neutrophil influx after direct ischaemia-reperfusion of the lung (Harada et al, 1994), where the mechanisms may be different.

4.5.4 Effect of ARIL.8.2 on neutrophil clearance from the pulmonary vasculature

As extensively discussed in the general introduction, neutrophil passage through the lungs is constrained by mechanical factors, and thus the neutrophils pass through the lungs relatively slowly. Most studies have found that because of this, some ^{111}indium labelled neutrophils are temporarily retained in the lungs after injection, and only gradually equilibrate with the circulating pool. The rate of decrease in the number of ^{111}indium labelled neutrophils in the lung was measured using external gamma scintigraphy. This showed that neutrophils were cleared relatively slowly from the right, pneumonic lung compared to the non-inflamed left lung. As discussed, neutrophil sequestration seems to be due primarily to rheological considerations, so this is likely to be secondary to a rapid increase in neutrophil stiffness as it traverses the inflamed lung, though changes in adhesion molecule expression (not only on the neutrophil, but also on vascular endothelium) could theoretically be involved.

The decreased clearance of neutrophils seen in the inflamed lung was inhibited by ARIL.8.2, so that clearance in the two lungs of ARIL.8.2 treated animals was not only equal

to each other, but also equal to that in the left, uninflamed lung of PBS treated control rabbits.

This shows the ARIL.8.2 was producing an effect, and this would be consistent with inhibiting IL-8 induced increases in neutrophil stiffness in the pneumonic lung. From this it can be inferred that the ARIL.8.2 utilised does indeed possess *in vivo* anti-IL-8 activity, as also demonstrated by its ability to inhibit neutrophil influx to the skin.

4.5.5 Immunohistochemistry

The anti-IL-8 antibody used in this study was a whole antibody. Thus, when it binds to IL-8 it is possible this would result in complement activation. This may be particularly relevant if IL-8 binds to the vascular endothelium in the lung, as *in situ* complement activation could then occur, leading to further inflammatory damage localised to the site of previous inflammation. Whilst IL-8 binding to endothelium has been found in other vascular beds but not in the lung (Rot et al, 1996), this possibility was investigated by immunohistochemistry. Goat anti-mouse immunoglobulin was used to detect tissue bound ARIL.8.2. None was discovered.

Activation of neutrophils by complement leads to their sequestration in the lung (Webster et al, 1982). Further, it has been shown that low levels of complement may act synergistically with other factors such as LPS to produce a more damaging response (Worthen et al, 1987). It is possible that more diffuse complement activation after ARIL.8.2/IL-8 binding acts in this way to cause neutrophil accumulation in the lung, increasing the neutrophils available for recruitment to the inflamed site.

Nevertheless, neutrophil influx in response to local generation of immune complexes in the skin, during the reverse passive Arthus response, was shown to be capable of inhibition by systemic anti-IL-8 antibody (Zhang et al, 1995) as has immune-complex mediated glomerulonephritis (Harada et al, 1994).

4.5.6 Plasma and broncho-alveolar lavage IL-8 levels

In a random selection of animals from each experimental group, plasma IL-8 levels were measure before antibody was administered. IL-8 was always undetectable at this time. Further measurements were also made on blood obtained immediately *post mortem*. In PBS treated control animals, levels were usually still undetectable. However, in ARIL.8.2 treated animals, there was an increase in plasma IL-8 seen in each of the four groups.

IL-8 levels were also measured in broncho-alveolar lavage fluid. Although IL-8 could be detected in some PBS controls, median levels were undetectable in PBS controls from each of the four groups. Levels were clearly higher in ARIL.8.2 treated animals than in the PBS treated controls.

This surprising finding is not directly explicable on the basis of available data. Supplied antibody had low endotoxin levels. It could result from intra-vascular complement activation resulting from antibody-antigen complexes, as discussed above. Immune complex formation could lead to further inflammation that in itself results in IL-8 formation.

If ARIL.8.2/IL-8 activates complement and leads to further IL-8 generation, this would be expected to be apparent whenever IL-8 and ARIL.8.2 are present simultaneously, but this has not been found to be true. Pigs injected with Pseudomonas aeruginosa developed high plasma IL-8 levels (up to 6 ng/ml). Free IL-8 levels were decreased after ARIL.8.2 to a maximum of 2 ng/ml or less (C. Hébert, personal communication).

If activation of complement is not responsible for the elevated IL-8 seen after ARIL.8.2, it might be hypothesised that IL-8 blockade interferes with negative feedback control of its production. This would lead to an increased production that would tend to overcome the effect of such a blockade, minimising its functional efficacy. Teleologically speaking, the body senses that 'not enough' IL-8 is being produced, and produces more to overcome this.

This sort of feedback control is not without precedence. For instance, a study of C5 sufficient and deficient mice challenged with Pseudomonas aeruginosa found that mortality was higher in deficient mice. Although neutrophil accumulation in the lungs was initially lower in the deficient mice, later neutrophil numbers were actually *higher* in the deficient mice (Larsen et al, 1982), indicating an adaptive response. A second example is seen in the action of anti-CD18 antibody on the neutrophil influx to the peritoneum after E. coli or thioglycollate broth (Winn and Harlan, 1993). As has been discussed in chapter three, this results in inhibition of neutrophil accumulation at 4 hours, but by 24 hours, neutrophil numbers had actually 'caught up', so that they were equal in antibody-treated animals and controls.

Negative feedback control of IL-8 production has not been demonstrated *in vitro.* Nevertheless, a wide variety of cells are capable of IL-8 production in response to a range of different stimuli, and it seems quite possible such control loops are established as part of local cytokine networks *in vivo*. It is notable that despite the fact that IL-8 *in vitro* causes selective neutrophil recruitment, injection of IL-8 into the joint space of rabbits produce full expression of the inflammatory response. There is initial neutrophil recruitment followed by mononuclear cell recruitment, before resolution occurs (Endo et al, 1991).

The potential for complex *in vivo* interaction of IL-8 is further emphasised by the realisation that its actions are not exclusively confined to the neutrophil. In the skin, for instance, it is also associated with angiogenesis, modulation of keratinocyte HLA-DR expression, proliferation, chemotaxis and Candida albicans killing, whilst IL-8 receptors are present on T-lymphocytes, macrophages, and mast cells (see Kemény et al, 1994). IL-8 production may well be part of much broader and more interactive processes than just being an 'on-off' signal for neutrophil influx, and this would suggest it is likely to be subject to subtle controls to ensure reliability and robustness of the system.

4.5.7 Comparison of antibody effects on neutrophil influx to pneumonia with other studies

IL-8 levels have been demonstrated to be elevated in the lung during bacterial pneumonia (Boutten et al, 1996, Chollet-Martin et al, 1993, and Villard et al, 1995), although there was no correlation between IL-8 levels and neutrophil numbers in broncho-alveolar lavage. Indeed, Villard et al note that IL-8 levels in broncho-alveolar lavage were similar in patients with pneumonia or ARDS, but the neutrophil influx was much greater in the former. Further, the finding of a correlation between neutrophil numbers and IL-8 levels in ARDS but not pneumonia raises the possibility that other chemotactic factors are important in pneumonia. The need for extreme caution in interpreting such correlations of neutrophil number and IL-8 levels has been discussed in the general introduction. However, the contention that factors additional to IL-8 are important in neutrophil influx during pneumonia is also supported by functional data. Broncho-alveolar lavage fluid from patients with pneumonia possesses chemotactic activity that can be split into three fractions. These were identified as C5a, LT B4 and a protein (presumably IL-8) with an approximate molecular weight of 10 000 (Hopkins et al, 1989).

The demonstration of elevated levels of IL-8 in broncho-alveolar lavage in this study is entirely compatible with the clinical studies quoted above.

Contradictory findings have been made on plasma IL-8 levels in pneumonia. They have been found to be unchanged (Boutten et al, 1996) or elevated (Chollet-Martin et al, 1993). The latter group was composed of patients requiring mechanical ventilation and hence may represent a subgroup with more severe disease. The finding in this study that plasma IL-8 was rarely raised in control animals with pneuS or pneuE is therefore in agreement with clinical findings.

The *in vitro* ability of IL-8 to cause neutrophil chemotaxis together with its presence in pneumonia, a condition characterised by the presence of abundant numbers of

neutrophils, suggests functional studies of its rôle in this disease would be appropriate. To date, none have been performed. The contribution of IL-8 to neutrophil influx to other sites and in other diseases has been assessed in a few studies using anti-IL-8 antibodies.

A number of studies have documented the ability of anti-IL-8 antibodies to inhibit neutrophil accumulation in the skin, in response to a variety of stimuli; intradermal IL-8, IL-1, local immune complex deposition in the reverse passive Arthus reaction (Zhang et al, 1995), intradermal LPS (Harada et al, 1993), and the tuberculin reaction (Larsen et al, 1995). Anti-IL-8 antibody has also been demonstrated to inhibit neutrophil accumulation in response to immune-complex mediated glomerulonephritis, LPS and IL-1 induced arthritis, and lung reperfusion injury (Harada et al, 1994) and endotoxin induced pleurisy (Broaddus et al, 1994). Neutrophil accumulation was also inhibited by anti-IL-8 antibody in response to hydrochloric acid instillation in the lung (Folkesson et al, 1995), whether the antibody was given prior to or 1 hour after the insult.

No studies documenting the failure of anti-IL-8 antibodies to inhibit recruitment of neutrophils to inflammatory sites have yet been published. Preliminary findings from one study of gut ischaemia-reperfusion show that although neutrophil influx locally to the damaged gut is inhibited by anti-IL-8 antibody, the antibody is without effect on the neutrophil accumulation to the lung that usually occurs following this insult (C. Hébert, personal communication). Although a publication bias against negative studies may partially account for this, other reasons must be sought.

As discussed above, the antibody ARIL.8.2 used in the current experiments has been shown in other studies to cause inhibition of neutrophil recruitment in other models. It was active as supplied when tested *in vitro*. Storage conditions of the antibody were not a factor as the antibody used for *in vitro* tests was taken from a randomly selected aliquot of a pool used for *in vivo* experiments, and was stored for a similar duration. Calculations suggest the dose should have been of sufficient magnitude, and higher doses had an effect that was no

different. The fact that it inhibited neutrophil influx to the skin, and prevented increased early neutrophil retention at the inflammatory site, demonstrate it was active *in vivo* during this study.

Taking the results at face value, several possible conclusions could be drawn. It is possible that neutrophil influx in pneumonia simply proceeds by mechanisms in which IL-8 is not essential, either because it usually has a minor rôle, or because other chemotactic factors such as C5a, LTB_4 or bacterially-derived products easily compensate. Their presence has been demonstrated in broncho-alveolar lavage fluid. Anti-IL-8 antibodies have been shown to inhibit neutrophil chemotactic activity of biological fluids from inflammatory sites by a maximum of 75 %, but often only 30 %, suggesting a contributory rather than dominant rôle for IL-8 in neutrophil chemotaxis *in vivo*.

Secondly, IL-8 may be under negative feedback control. Anti-IL-8 antibody could inhibit feedback, giving rise to generation of extra IL-8 (reflected in increased plasma and broncho-alveolar lavage IL-8 levels), sufficient to overcome the blockade. Why either of these first two mechanisms should occur in this model and not others is not apparent, and one would have to hypothesise mechanisms unique to pneumonia.

Thirdly, binding of IL-8 to anti-IL-8 antibody may cause complement activation either throughout the vasculature, locally on the vessel wall, or in the interstitium, at the site of inflammation, any of which could cause neutrophil recruitment. Although no direct evidence for such complement activation was obtained, the fact that complement activation causes neutrophil sequestration specifically in the lung may account for the difference between both this study and the preliminary findings of selective lack of efficacy of anti-IL-8 antibody on neutrophil influx to the lung after gut ischaemia-reperfusion, compared to other studies in other sites. It also provides an explanation for the raised plasma and broncho-alveolar lavage IL-8 levels seen after antibody administration. The fact that anti-IL-8 antibody was able to block immune complex-mediated neutrophil influx to the skin and

the kidney is not necessarily relevant given the particular ability of complement activation to cause neutrophil accumulation in the lung.

Fourthly, there may be a general difference in the requirement for IL-8 in areas supplied by pulmonary and vascular circulations, in a manner analogous to the differing requirement for CD18 in the two territories (Doerschuk et al, 1990, and Hellewell et al, 1994). The fact that ARIL.8.2 has been used to successfully inhibit neutrophil accumulation to the lung makes this argument less likely (Folkesson et al, 1995).

Fifthly, the timing of administration of the antibody differed in this study from all others. It was administered after either 6 or 30 hours in this study, whereas in all other studies (except one in which it was administered one hour after the insult) the antibody has been administered prior to the challenge. If IL-8's importance in neutrophil influx is confined to the early part of the evolution of inflammation, and other factors then supersede, then the lack of effect on neutrophil influx in the current study combined with the inhibitory effect seen in other studies would be expected. The change in functional importance of IL-8 with time has only been addressed once. It was found to have increased importance after 2 hours, as assessed by the ability of anti-IL-8 antibody to inhibit the chemotactic activity of peritoneal fluid, (as assessed in the back skin of assay rabbits), obtained at intervals after intraperitoneal injection of zymosan (Collins et al, 1991). Nevertheless, this may be different in other situations. Unpublished work also shows that the functional rôle of IL-8 may change with time. Replication deficient adenoviruses induce prolonged expression of IL-8 in primates as assessed by sequential broncho-alveolar lavage over several weeks, whereas neutrophil accumulation is transitory, lasting several days at most (R. Wilmott, personal communication).

Sixthly, the current study measures specific neutrophil influx whereas the other studies measure neutrophil numbers. The possibility that neutrophil numbers decrease after anti-IL-8 because of an increase in clearance rather than a decrease in influx is unlikely,

given the known *in vitro* neutrophil activating and chemotactic actions of IL-8. *In vitro* IL-8 has no effect on neutrophil apoptosis (Colotta et al, 1992). Indeed, chapter four presents results showing broncho-alveolar lavage interleukin-8 levels correlate weakly with total neutrophil apoptosis rather than demonstrating the inverse relationship this explanation would suggest.

Finally, the current study is the only one performed with live bacteria. Although studies with nebulised bacteria suggest after 4 hours only 7.3 % of Strep. pneumoniae are viable, whilst 88.5 % of E. coli are viable (Jay et al, 1976), after bolus challenge, viability may be greater (Ansfield et al, 1977). At least in the 6 hour group, there may well be viable bacteria in the lung when anti-IL-8 antibody is introduced. Any reduction in neutrophil influx and activation could give rise to increased survival, providing a further stimulus to inflammation, mitigating the inhibitory effects of the antibody.

The apparent lack of effect of the anti-IL-8 antibody on neutrophil influx in pneumonia may therefore be because IL-8 is not of over-riding importance in the lung during pneumonia, or that its importance is confined to the first 6 hours of disease. Alternatively, it could be secondary to complement generation, or interference with positive feedback controls. The most likely explanations in view of the raised IL-8 levels in plasma and broncho-alveolar lavage fluid after the administration of antibody, coupled with the trend to an increased influx after antibody rather than merely absence of inhibition, are that anti-IL-8 antibody results in activation of complement giving secondary neutrophil influx, or that there is release of a negative feedback mechanism.

4.6 Summary

1) ARIL.8.2 was able to inhibit IL-8 induced neutrophil shape change *in vitro.*

2) ARIL.8.2 was able to inhibit neutrophil influx in response to intradermal IL-8 *in vivo.*

3) Administration of 2 mg/kg ARIL.8.2 prior to administration of ^{111}indium-labelled neutrophils failed to produce any reduction of influx at either 6 or 30 hours in pneuS and pneuE. Indeed there was a trend to increased influx. It was however able to inhibit the increased early retention of In-wbc in the inflamed lung, as assessed by external gamma scintigraphy.

4) Administration of 6mg/kg of ARIL.8.2 produced identical results.

5) Immunohistochemistry failed to demonstrate localisation of ARIL.8.2 to the pulmonary vascular epithelium, failing to support the contention that endothelially bound IL-8 could be involved in immune complex generation.

6) Administration of the ARIL.8.2 in animals with pneumonia was associated with increased plasma and broncho-alveolar lavage IL-8. This may reflect immune complex formation between ARIL.8.2 and IL-8, giving complement activation and further IL-8 generation, or the release of normal feedback inhibition of IL-8 formation.

7) In view of the increased plasma and broncho-alveolar lavage IL-8 levels after IL-8 administration, it is considered that the lack of ability of ARIL.8.2 to inhibit neutrophil influx in pneumonia is most likely to be secondary to interference with a normal *in vivo* feedback control of IL-8 production. Other possible mechanisms are discussed, and although there is some evidence against it, it is possible that ARIL.8.2/IL-8 immune-complex formation is important.

5. THE ROLE AND CONTROL OF APOPTOSIS IN NEUTROPHIL CLEARANCE IN PNEUMONIA

5.1 Introduction

Neutrophils are recruited to the lungs in vast numbers during pneumonia. This places the lung in a potentially precarious position, given the array of potentially histotoxic products associated with neutrophils (Haslett et al, 1989, Henson and Johnson, 1987, Root and Cohen, 1981). It is remarkable that in pneumococcal pneumonia (Johnston, 1991), restoration of lung architecture is usually prompt and complete. This implies a safe and efficient clearance mechanism for neutrophils. In gram negative pneumonia there is often some disruption of pulmonary structure (Coalson, 1995). As discussed in chapter two, there is a more prolonged influx of neutrophils to the right lung in pneuE than pneuS, and this may give an increased lung neutrophil burden, contributing to lung damage. Manipulations of neutrophil number with G-CSF or cyclophosphamide have supported this view that some of the lung damage produced in gram negative pneumonia is secondary to neutrophils rather than being a directly due to bacteria (Terashima et al, 1995).

Neutrophils have never been observed to return to the circulation once they have left it, so they must meet their fate in the tissues. As discussed in the general introduction, it was long presumed there was passive degeneration of the neutrophils. An alternative, much less hazardous explanation is provided by the finding that neutrophils are able to undergo apoptosis (programmed cell death) (Savill et al, 1989). *In vitro* experiments have shown that after this process, macrophages can the engulf the neutrophil without generation of further inflammatory mediators (Meagher et al, 1989). Studies have suggested that this accomplished by a mechanism using the vitronectin receptor and the thrombospondin

receptor on the macrophage, with thrombospondin forming a 'bridge' to the neutrophil (Savill, 1992), or by a process inhibitable by phosphatidylserine (Fadok et al, 1992).

The presence of apoptotic neutrophils and macrophages that have ingested apoptotic neutrophils in broncho-alveolar lavage fluid after pulmonary LPS challenge suggests strongly that this mechanism could be important in pneumonia (Cox et al, 1995). A large number of inflammatory mediators affect the rate of neutrophil apoptosis *in vitro,* suggesting that this process could be actively controlled *in vivo*. Inflammatory mediators (such as GM-CSF, G-CSF, C5a, FMLP, IL-6 and IFN-γ) have usually been shown to delay apoptosis, as have bacterial components such as inactivated Streptococci and LPS (referenced in general introduction). Generally, it has been considered likely that these act at the inflammatory site in order to prolong neutrophil lifespan there, thus prolonging its ability to perform defensive actions (Haslett, 1992). Without specific modulatory instruction via inflammatory mediators, the neutrophil will be programmed to have an early and safe end to its life via apoptosis.

It was therefore decided to seek evidence for apoptosis during pneuS and pneuE by quantifying neutrophil numbers in broncho-alveolar lavage fluid. The presence of alveolar macrophages with apoptotic bodies was also sought. The ability of broncho-alveolar lavage fluid to modulate the rate of apoptosis of neutrophils *in vitro* was then examined as an indirect way to measure the modulatory effect of the inflammatory environment of the pneumonic lung.

5.2 Methods

Pneumonia was induced as in previous experiments. 6 animals were initially examined at 0 (unbronchoscoped control), 12, 24, 48, 72 and 96 hours. Following these preliminary experiments, 6 further animals were studied at 0, 12, 24 and 48 hours. Formal statistical analysis was performed on the complete data sets where n = 12, but data from the

remaining two data points where n = 6 are presented graphically for comparison. It should be noted that timings referred to in this chapter refer to actual time after the induction of pneumonia, not the time between induction of pneumonia and injection of In-wbc, as referred to in other chapters.

Animals were killed with an intravenous overdose of pentobarbital. The great vessels were sectioned intra-abdominally and the heart and lungs removed *en bloc*. The pulmonary vasculature was flushed twice with 30 ml of normal saline introduced via a 19 G hypodermic needle within the right ventricle. The number of apoptotic cells recovered was expected to be low for reasons examined in the discussion. For this reason, selective broncho-alveolar lavage of the right cranial and medial lobes was performed, rather than lavage of the entire right lung. Cells from the area of active inflammation would be far less 'diluted' by cells from the remaining lung, a consideration far less important when using radiolabelled cells, when the technique is 'blind' to the presence of unlabelled cells. The left main bronchus was tied off with 2/0 Mersilk as before. Connective tissue was dissected away from the right main bronchus to expose the right caudal lobe bronchus which was ligated. A portion of tubing from a wide bore extension set was tied in place in the trachea. As much as possible of a 10 ml aliquot of PBS (without cations) with 50 U of heparin per ml was introduced, then aspirated, and this was repeated twice. The whole procedure was repeated with a further 10 ml aliquot. Broncho-alveolar lavage was kept on ice until further processing.

The lungs were inflated to 25 cm. of water pressure with 10 % (w/v) paraformaldehyde in PBS, and fixed overnight. They were then sectioned for light microscopy.

Cytocentrifuge preparations were prepared in quadruplicate using 100 µl of broncho-alveolar lavage for each. These were stained with Diff-Quik®. Differential counts were

performed on at least 500 cells per slide. In particular, the number of alveolar macrophages containing large, chromatin inclusions representing apoptotic bodies was counted and expressed as a percentage of the total alveolar macrophage numbers.

Apoptotic rabbit neutrophils were found to retain their morphology unreliably on cytocentrifuge preparation. Cells were therefore assessed for apoptosis in suspension using acridine orange staining. 6 µl of 40 % formaldehyde was added to a 100 µl of broncho-alveolar lavage sample, and saved at 4°C for later acridine orange staining from which differential counts of all macrophages (with and without inclusions) and all neutrophils (apoptotic or non-apoptotic) were obtained.

Erythrocytes were lysed by the addition of 4 drops of Zap-0globin (Coulter Electronics, Luton) to a further 100 µl of broncho-alveolar lavage and Isoton was added to 10 ml. A Coulter counter (Coulter Counter® ZM with Sampling Stand II_a) was used to count cells, and total cell recovery calculated.

A 25 µl aliquot of 25% glutaraldehyde (electron microscopy grade, BDH Laboratory Supplies, Poole) was added to a further 200 µl sample of broncho-alveolar lavage, and stored at 4°c for later preparation for electron microscopy.

In view of the fact that at some time points the percentage of neutrophils in broncho-alveolar lavage was quite low, and that apoptotic cells were expected to be a small fraction of this, neutrophils were concentrated for further assessment. The remaining broncho-alveolar lavage was centrifuged at 250g for 6 minutes. The cell pellet was resuspended in 10 ml of a 6:4 mixture of hetastarch (Hespan®, Geistich Pharma, Chester) and 90 % Percoll in 10 x Hanks Buffered Salt Solution without cations. This was transferred to a 15 ml polyethylene centrifuge tube, and centrifuged at 2500g for 10 minutes.

Following this, macrophages were found on the surface of the hetastarch:Pecoll mixture. They were aspirated together with much of the supernatant. On the bottom of the tube were the erythrocytes, and the neutrophils were mixed with and just above this layer. (A test of this gradient with a mixture of 50 % alveolar macrophages and 50 % neutrophils showed the upper layer to contain 97.8 % macrophages and the lower layer to contain 96.8 % neutrophils).

The neutrophil layer was aspirated and washed once with phosphate buffered saline without cations, before being resuspended in 200µl of PBS. Half of this was added to 25 µl of 25% glutaraldehyde, and half added to 6 µl of 40 % formaldehdye. These were stored at 4°C. The former was later processed for electron microscopy. The latter was stored for later acridine orange staining, and the per centage of cells that were apoptotic were counted.

Meanwhile, the broncho-alveolar lavage fluid supernatant was centrifuged at 2500g for 10 minutes at 4°C. The supernatant was then saved in 1.5 ml Eppendorf tubes at -70°C until further use.

Acridine orange staining was performed by adding 1 µl of acridine orange (1 mg/ml in PBS) to 10 µl of cell suspension. Examination of cells was performed using fluorescence microscopy. At least 500 cells were counted per specimen, with the exception that so few neutrophils were obtained from unbronchoscoped animals that only 100 - 200 cells (and occasionally even fewer) could be counted. Apoptotic cells were recognised by their uniformly staining, compacted chromatin, often with separation of the lobes of the nucleus, in shrunken cells.

5.2.1 Electron microscopy

Material for electron microscopy was kindly prepared by Mr. S. McKenzie, Dept. of Pathology, Edinburgh University Medical School using reagents obtained from TAAB

Laboratories Equipment Ltd., Aldermaston. Fixed cells were washed in sodium cacodylate buffer. They were post fixed in 1 % osmium tetroxide in sodium cacodylate buffer for 2 hours, then dehydrated through grades of ethanol to 100 %. They were impregnated in Araldite epoxy resin overnight, before embedding in fresh Araldite epoxy resin. This was polymerised at 56°C for 3 days.

Ultrathin, 60 nm sections were cut on a LKB Nova ultrotome. Sections were stained with uranyl acetate and lead citrate.

Sections were viewed on a Philips CM14 Electron Microscope.

5.2.2 In vitro *experiments using rabbit neutrophils*

The ability of broncho-alveolar lavage fluid supernatant to modulate apoptosis of rabbit neutrophils cultured *in vitro* was assessed. Neutrophils were prepared as described and resuspended at 4 x 10^6/ml in Iscove's Modified Dulbecco's Medium, supplemented with 20 % normal rabbit serum, and 100 U/ml penicillin and 200 mg/ml streptomycin. A 100 µl aliquot of cell suspension was placed in the wells of a 96 well tissue culture plate, 9 wells being set up per assay condition. 100 µl of PBS without cations, with 50U per ml heparin (negative control), or 100 µl of broncho-alveolar lavage fluid supernatant was added to each set of 9 wells. Experiments were arranged so that lavages from a series of animals receiving bacteria from the same pool were assayed together using neutrophils from a single pool.

The neutrophils were incubated at 37°C in 5 % CO_2. After 5, 10 and 20 hours, cells from two wells for each condition were removed and fixed by the addition of 6 µl 40 % formaldehyde. The cells were stained with acridine orange, and the percentage of neutrophils that were apoptotic assessed by counting at least 500 cells on a fluorescent microscope (Olympus Optical Co., London). The mean of the duplicates was used for data presentation. A 50 µl aliquot of cell suspension was taken from a further well and used to

measure cell recovery with a Coulter counter. Recovery of cells was around 70 % and did not vary between groups. A 20 µl aliquot of cell suspension was added to 20 µl of Trypan Blue, and the percentage of cells able to exclude the dye was counted as a measure of viability. Cells obtained at 20 hours showed high rates of Trypan Blue positivity and were excluded from further assessment. At earlier time points > 95 % (and usually > 98 %) were able to exclude Trypan Blue dye.

Data is presented as the ratio of apoptosis produced in a given condition to apoptosis produced by PBS/heparin control.

5.2.3 Chemokine assays

Measurement of IL-8 in broncho-alveolar lavage fluid was kindly performed by Genentech Inc., San Francisco, USA, as described in chapter 4.

Gro and MCP-1 were kindly measured by O. Kajikawa in the laboratory of T. Martin, Seattle, using ELISAs.

5.2.4 In vitro experiments using human neutrophils

Similar experiments to those performed with rabbit neutrophils were performed with human neutrophils, with the exception that assessment was performed at 6 and 20 hours, and used Diff-Quik®-stained cytocentrifuge preparations instead of acridine orange stained cell suspensions, as they produced clear morphology with this technique. Slides were kindly counted by Dr. S. Hannah, Rayne Laboratory, Edinburgh, blinded to treatment, with at least 500 cells being classified for the presence or absence of apoptosis per slide. Data is presented as for data with rabbit neutrophils.

5.2.5 Inhbition of in vitro *apoptosis using blocking antibodies*

Preliminary experiments were performed to establish whether blockade of the neutrophil Fas or soluble TNFα dimished the pro-apoptotic effect of broncho-alveolar lavage fluid.

Broncho-alveolar fluid samples were selected on the basis of their demonstrated ability to induce high levels of apoptosis. Anti-TNF (demonstrated to inhibit rabbit TNFα in L929 cytotoxicity assays using supernatant from endotoxin-stimulated rabbit alveolar macrophages as a source of TNFα) was obtained via R&D and was preincubated with bronchoalveolar lavage fluid to a concentration of 10 μg/ml for 30 minutes. The anti-human Fas antibody CH11 (Upstate Biotechnology) was preincubated at 0.5 μg/ml with human neutrophils for 30 minutes prior to the addition of broncho-alveolar lavage fluid. Apoptosis after 6 or 20 hours as assessed on Diffquik staining was then compared to apoptosis in the abscence of antibody, or after preincubation with 7F4, a control antibody (an anti-CD44 antibody, courtesy of I. Dransfield) with broncho-alveolar lavage fluid or neutrophils (as appropriate), or incubation with 12.5 ng/ml TNFα as a positive control. The anti-TNF antibody used was able to completely inhibit the effect of this dose of TNF.

5.3 Discussion of methods

Pneumonia was induced as in experiments described in chapter 2, and as discussed there, this reliably produces a localised, intense inflammation, showing the expected progression from neutrophil to mononuclear predominance, with progression to resolution.

Standard, established methods were used to assess apoptotic morphology on light microscopy and electron microscopy (Wyllie et al, 1980, Kerr et al, 1987, Payne et al, 1994). On light microscopy, neutrophils were shrunken, with dense chromatin condensation, frequently accompanied by a degree of nuclear fragmentation. On electron microscopy, apoptotic neutrophils displayed characteristic smoothness of the cell membrane with a lack

of pinocytic vesicles, dilatation of the endoplasmic reticulum, and dense condensation of the chromatin. Acridine orange staining has been successfully used to study apoptosis in a variety of leukocytes (Arends et al, 1990, Stern et al, 1992 and Bellingan et al, 1994). Apoptotic cells appear small, and the nucleus is seen to be contracted, with uniform, intense staining of the chromatin, rather than the normal diffuse pattern.

Many publications relating to apoptosis have included biochemical confirmation of apoptosis by DNA gel electrophoresis and demonstration of a 'ladder' pattern of DNA fragments produced by internucleosomal cleavage. This is applicable to cell culture studies, where large numbers of cells may proceed to apoptosis without the presence of macrophages to clear them. However, in tissue, apoptotic cells are generally cleared rapidly, so that even with a significant clearance of cells by apoptosis, free apoptotic cells are only rarely found because of prompt clearance. DNA electrophoresis will not produce clear results in these circumstances, so was not attempted in these studies.

Although only morphological assessment is used here, this should be fully adequate. Neutrophils showing morphological apoptosis, but not showing associated biochemical features of apoptosis, have never been described. It should also be remembered that the original definition of apoptosis was based on a morphological classification of modes of cell death (Kerr et al, 1972). Thus, morphological appearances are sufficient to confidently classify a cell as apoptotic.

5.4 Results

5.4.1 Light microscopy of tissue

Light microscopy of lung tissue from animals with pneumonia clearly demonstrated that some neutrophils showed the classical morphological appearances of apoptosis (see Figure 5-1). In addition, alveolar macrophages were seen that contained apoptotic neutrophils in varying degrees of degradation (see Figure 5-2).

Figure 5-1 Apoptotic neutrophils seen on tissue sections

The photomicrographs show clear examples of apoptotic neutrophils (arrowed) in the alveolar spaces during pneumonia (H&E, om x1000)

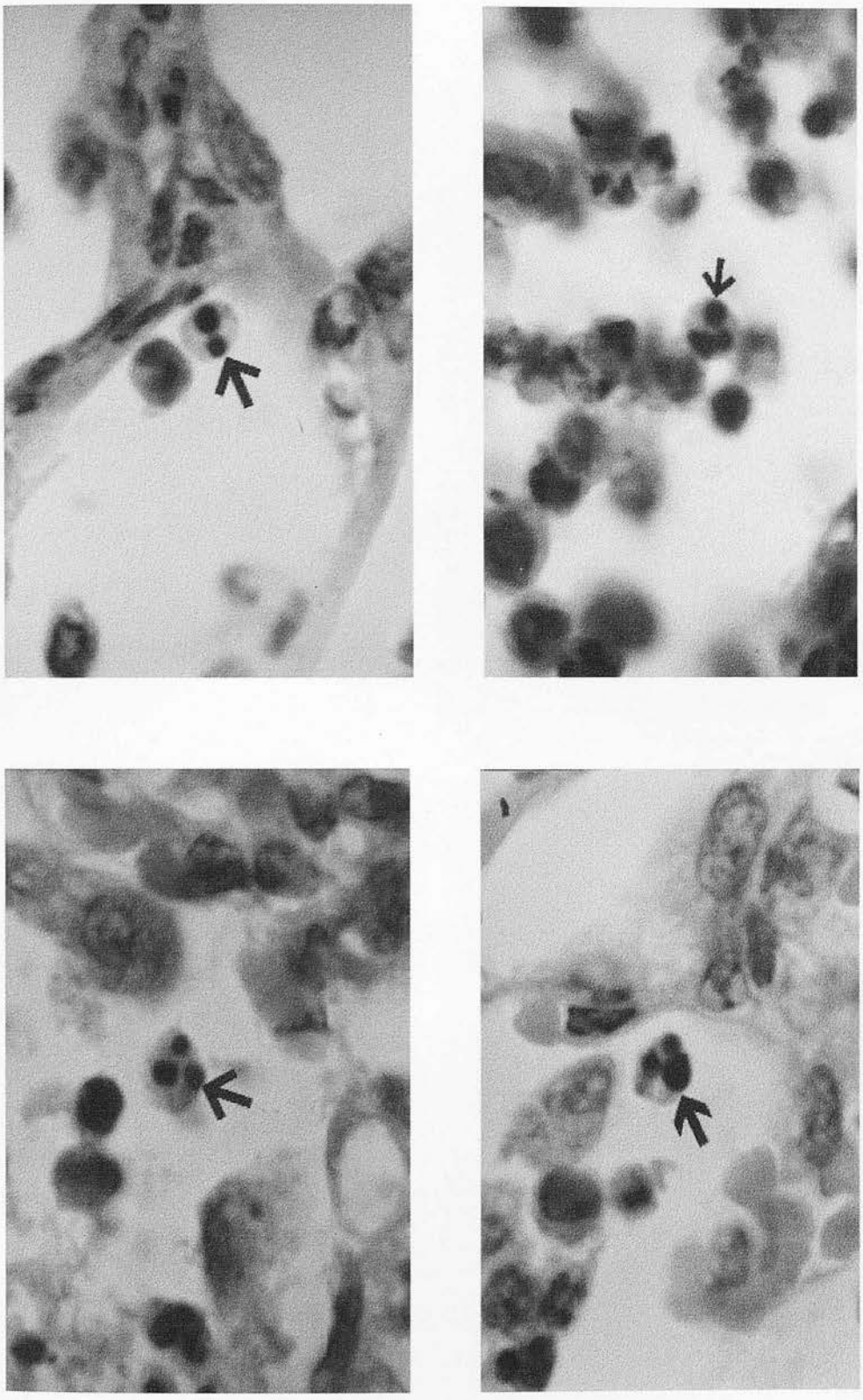
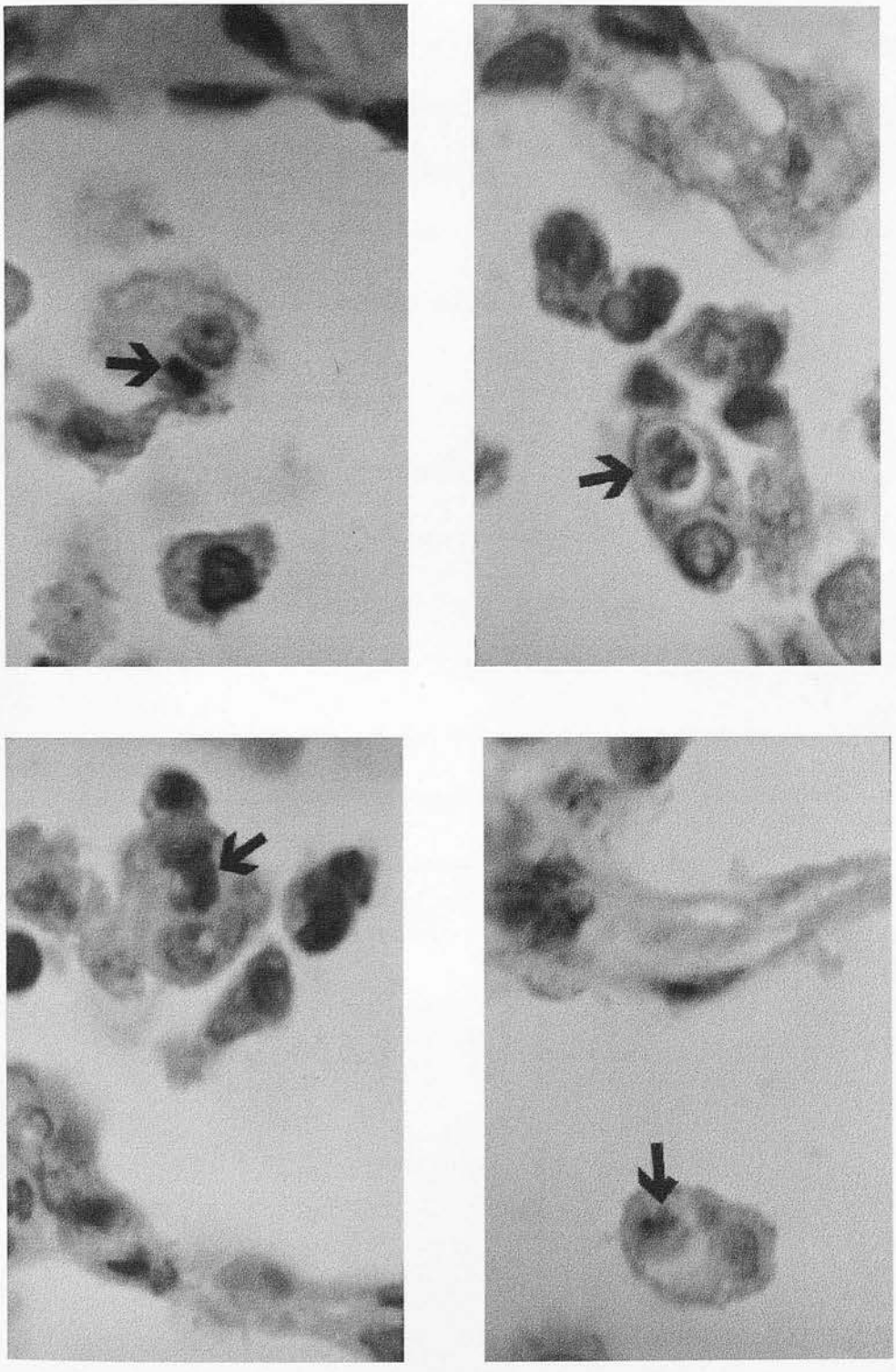


Figure 5-2 Alveolar macrophages containing apoptotic neutrophils, seen on tissue sections

The photomicropgraphs show alveolar macrophages with vacuoles showing apoptotic neutrophils (arrowed) in varying stages of degradation (H&E, om x1000).



5.4.2 Cellular constituents of bronchoalveolar lavage

5.4.2.1 Presentation of results

Initially, 6 animals were studied at the following time points; 0, 12, 24, 48, 72 and 96 hours. Examination of these preliminary time points suggested that changes in apoptotic cell recovery and number of macrophages containing apoptotic bodies was greatest between the first four of these points. A further 6 rabbits were therefore examined at these time points. Statistical analysis is performed for 0, 12, 24, and 48 hours, with n = 12 per group. Graphs include data for 72 and 96 hour groups for comparison and examination of trends, but these are not included in formal statistical analysis.

5.4.2.2 Morphological appearances

Light microscopy using Diff-Quik® -staining of BAL cytocentrifuge preparations, and acridine orange-staining of concentrated neutrophils from BAL, showed morphological evidence of apoptosis (see figure Figure 5-3 and Figure 5-4) in both types of pneumonia. In addition, light microscopy of Diff-Quik® stained cytocentrifuge preparations showed evidence of alveolar macrophages that had ingested apoptotic neutrophils, and these were present within vacuoles in varying degrees of degradation (see Figure 5-5)

Electron microscopy confirmed both of these findings (see Figure 5-6 and Figure 5-7).

Figure 5-3 Neutrophils showing apoptosis from BAL (Diff-Quik®)

The photomicrographs show examples of neutrophils from BAL showing morphological features of apoptosis (arrowed). (Diff-Quik®, om x1000).

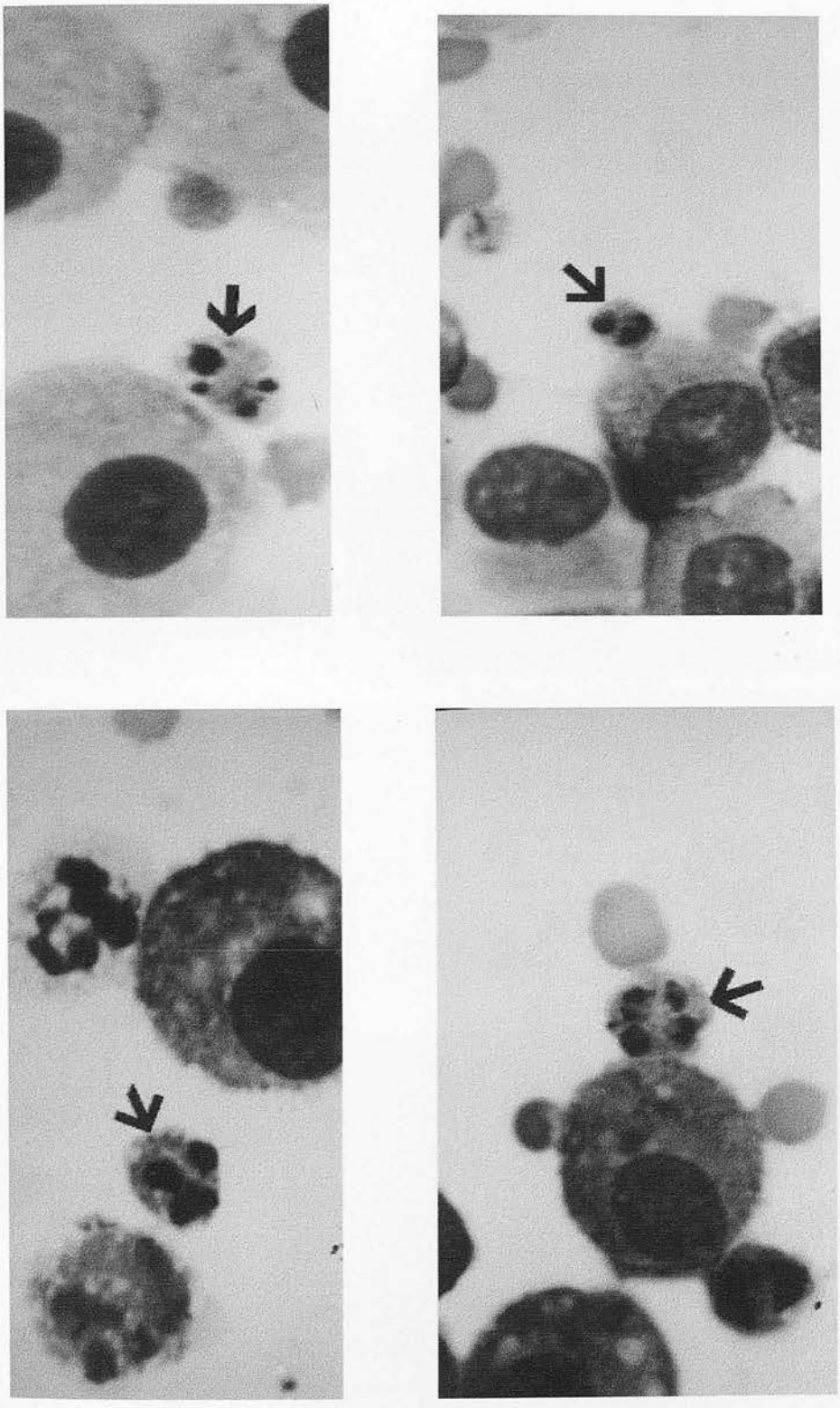


Figure 5-4 Neutrophils showing apoptosis from BAL (Acridine orange)

The photomicrographs show neutrophils with the morphological appearances of apoptosis (arrowed). Although the photomicrographs show the condensed, smooth, featureless chromatin of apoptotic cells, this is much clearer during microscopy. As they are examined in suspension, it is necessary to continually alter the plane of focus to appreciate their morphology fully. (Acridine orange, om x400).

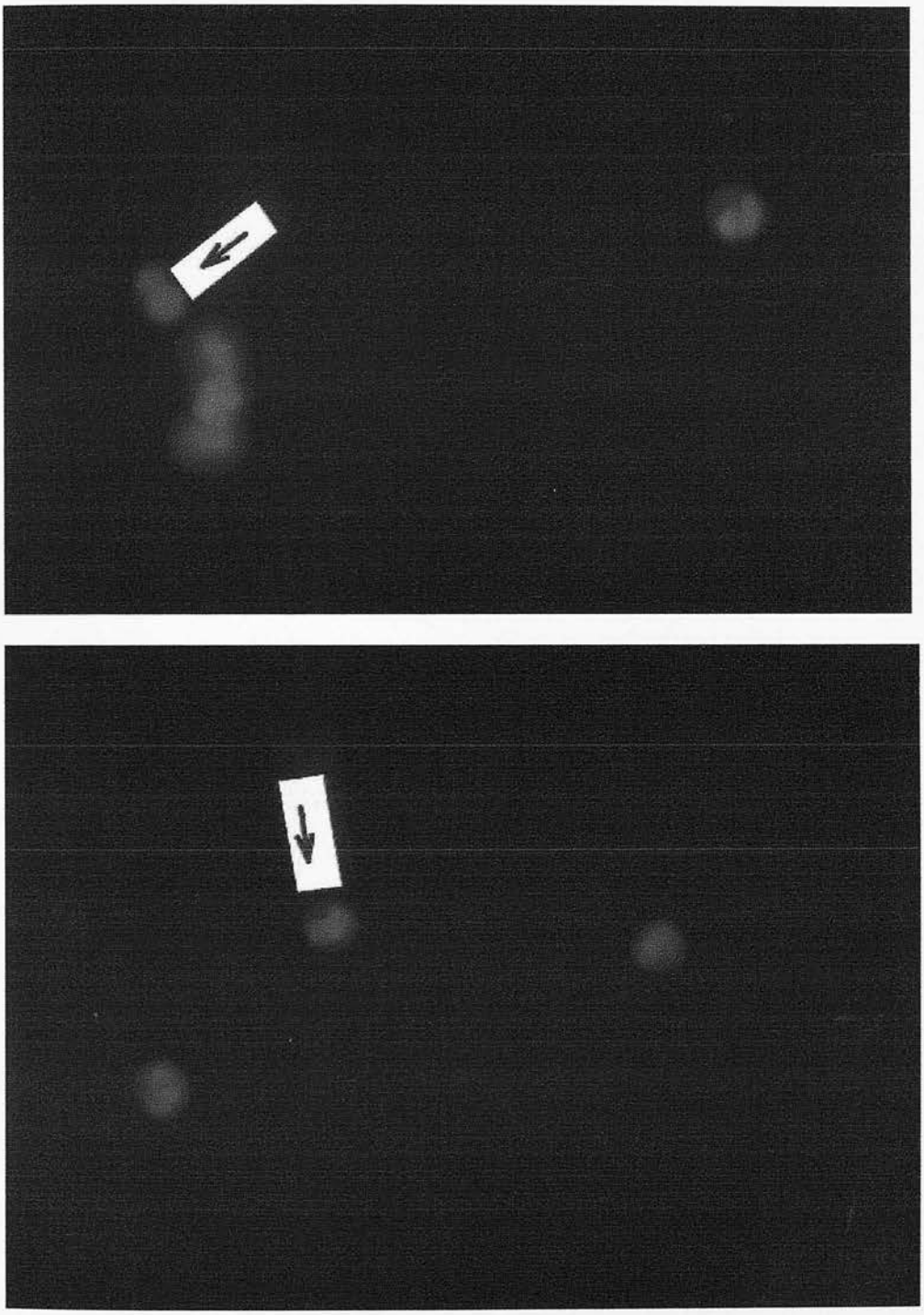


Figure 5-5 Alveolar macrophages containing apoptotic bodies from BAL

The photomicrographs show examples of alveolar macrophages from BAL, containing apoptotic neutrophils with varying amounts of degradation (arrowed). (Diff-Quik®, om x1000).

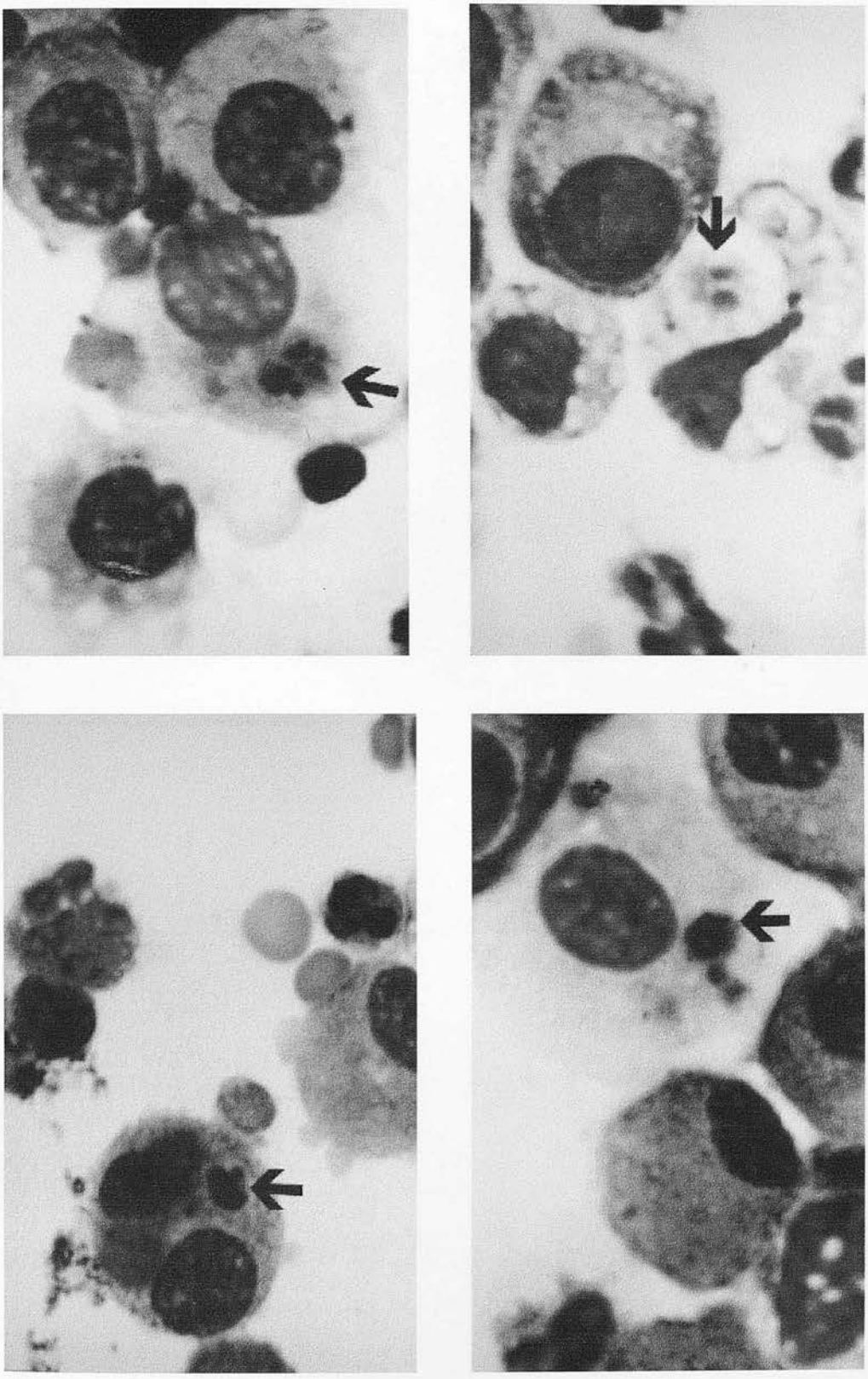


Figure 5-6 Electron microscopy of neutrophils showing apoptosis from BAL

The electron-micrographs show the appearances of neutrophil recovered from BAL. The upper panel shows a neutrophil showing early apoptosis. It has a smooth outline. One of the nuclear lobes has condensed chromatin, but the others retain normal structure, including pale euchromatin. The bottom pair of electron micrographs show shrunken apoptotic cells with smooth, featureless membranes and condensed, uniform chromatin. (om x 9 600).

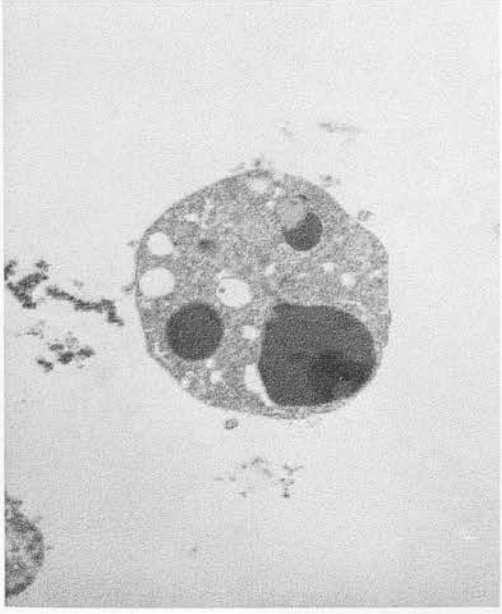
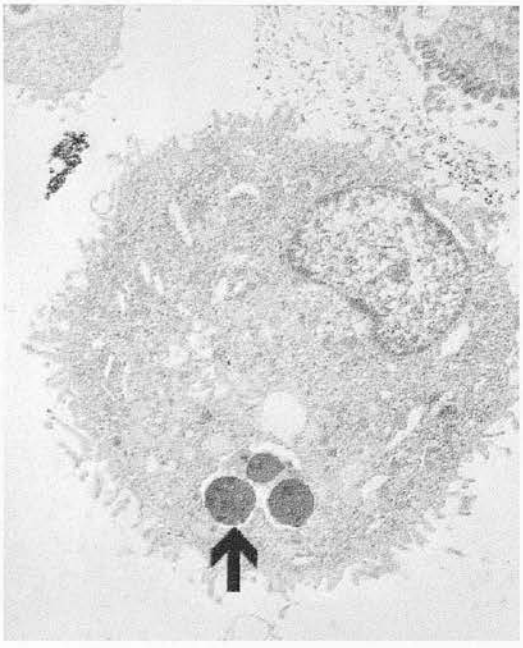
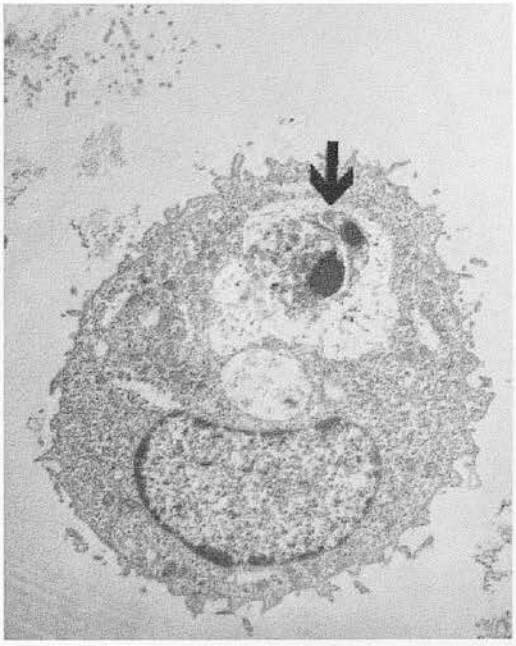


Figure 5-7 Electron microscopy of alveolar macrophages from BAL showing vacuoles containing apoptotic neutrophils

The electron micrographs show alveolar macrophages with vacuoles containing partially degraded apoptotic neutrophils (arrowed). (om x5600).

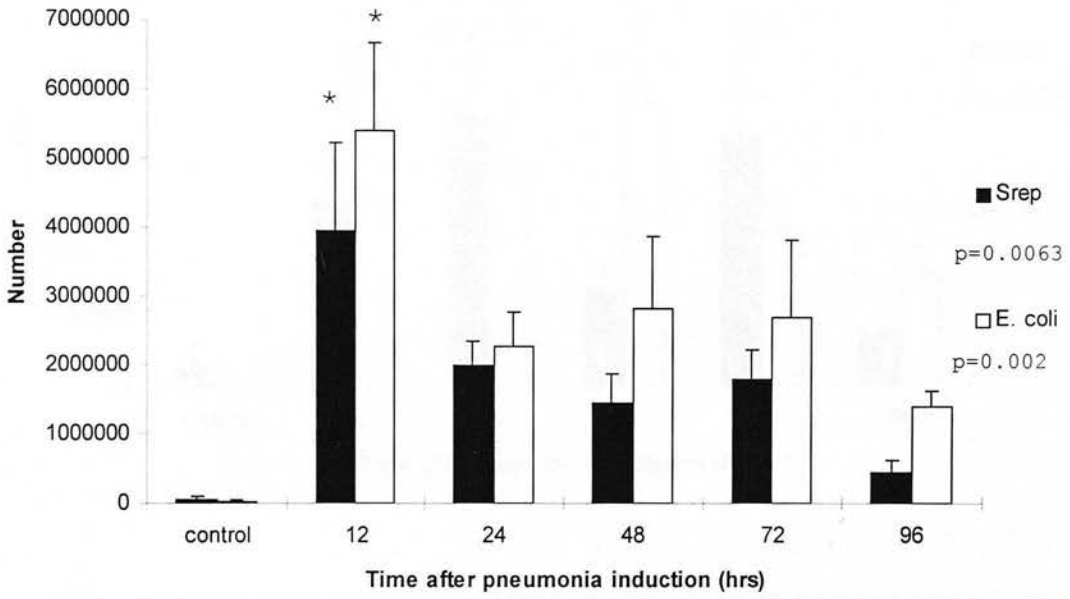




5.4.2.3 Total recovery of neutrophils from broncho-alveolar lavage

Figure 5-8 Recovery of neutrophils from broncho-alveolar lavage

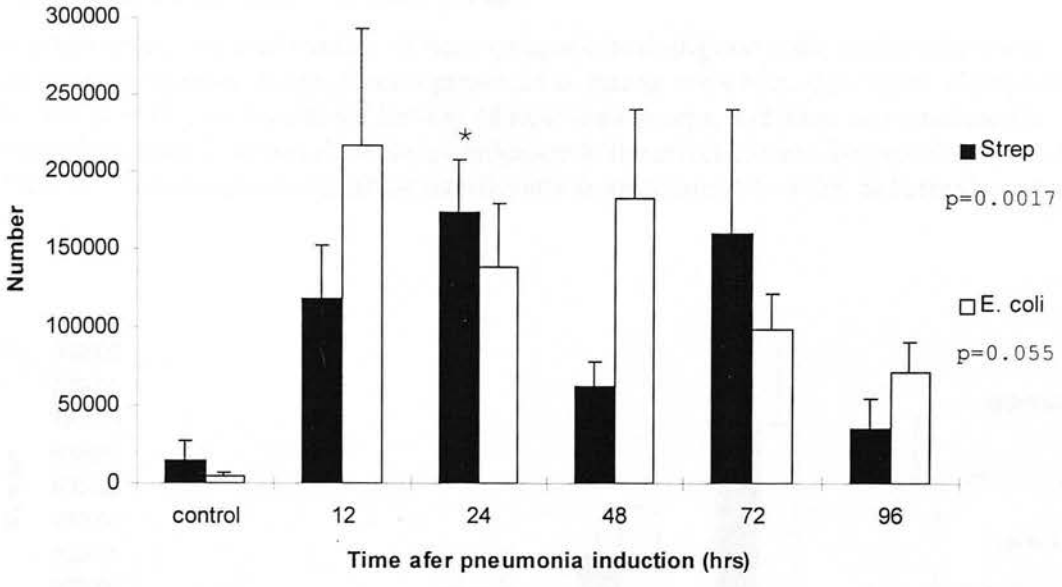
The graph shows the total number of neutrophils recovered from broncho-alveolar lavage. Data is presented as means, error bars show SEM. Complete data sets (n = 12) were obtained for 0 to 48 hour time groups, and these were included in statistical analysis. P values show the significance of the effect of time for pneuS and pneuE (ANOVA). * shows groups differing significantly from control (P < 0.05, Scheffe's *post hoc* test).



In control animals the recovery of neutrophils by selective bronchoalveolar lavage of the right cranial and middle lobes was very low ($5.5 \times 10^4 \pm 0.4 \times 10^4$ and $3.4 \times 10^4 \pm 0.5 \times 10^4$ in the two groups). ANOVA of groups with complete data sets (i.e. control, 12 hour, 24 hour and 48 hour for which n = 12) showed a significant effect of time in both pneuS and pneuE ($p = 0.0063$ and $p = 0.002$ respectively). *Post hoc* analysis showed that only the 12 hour group had a significant elevation of neutrophil numbers above control levels, rising about 80 to 100 fold in the two types of pneumonia. The trend was for a high early peak at 12 hours followed by a decline, which implies neutrophil clearance has begun between 12 and 24 hours.

Figure 5-9 Recovery of apoptotic neutrophils from broncho-alveolar lavage

The graph shows the total number of apoptotic neutrophils recovered from broncho-alveolar lavage. Data is presented as means, error bars show SEM. Complete data sets (n = 12) were obtained for 0 to 48 hour time groups, and these were included in statistical analysis. P values show the significance of the effect of time for pneuS and pneuE (ANOVA). * shows groups differing significantly from control ($P < 0.05$, Scheffe's *post hoc* test).



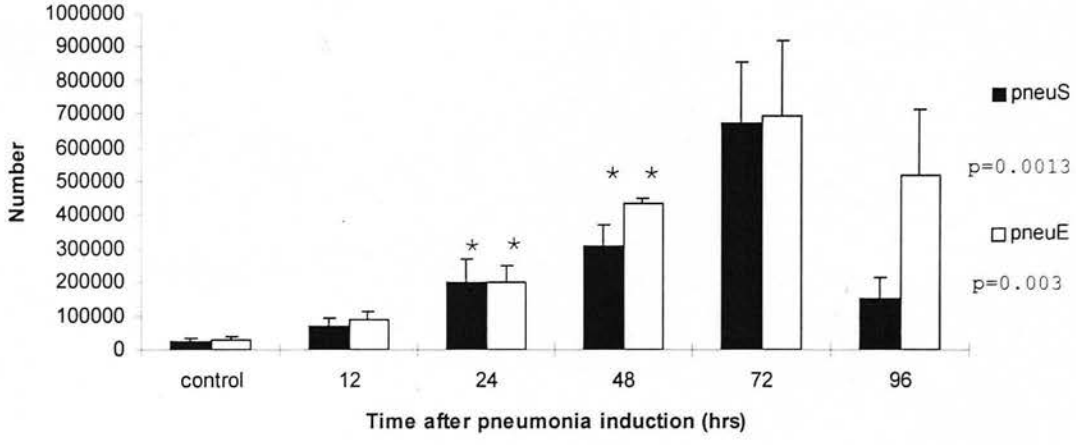
Right cranial and middle lobe bronchoalveolar lavage recovered very few apoptotic neutrophils from control animals ($1.5 \times 10^4 \pm 1.3 \times 10^4$ and $0.5 \times 10^4 \pm 2\ 0.2 \times 10^4$, mean $\pm$ SEM in the two control groups). In pneuS there was a significant effect of time on recovery of apoptotic neutrophils ($p = 0.0017$) but this just failed to reach significance in the pneuE group ($p = 0.055$). *Post hoc* analysis showed that the 24 hour pneuS group was the only one in which apoptotic neutrophil recovery was significantly greater than control, with $17.4 \times 10^4 \pm 3.4 \times 10^4$ (mean $\pm$ SEM) being recovered. It is notable that this peak of apoptotic neutrophil recovery follows the peak in total neutrophil recovery, and indeed the overall trend is for a later, more sustained rise in numbers of apoptotic neutrophils compared to total neutrophils, as expected if neutrophils are ageing *in situ* and becoming apoptotic.

Overall, a similar pattern is seen in pneuE, even though the changes just fall short of reaching significance.

5.4.2.5 Total alveolar macrophages containing apoptotic bodies recovered from broncho-alveolar lavage

Figure 5-10 Total number of macrophages containing apoptotic bodies recovered from broncho-alveolar lavage

The graph shows the total number of macrophages containing apoptotic bodies recovered from broncho-alveolar lavage. Data is presented as means, error bars show SEM. Complete data sets (n = 12) were obtained for 0 to 48 hour time groups, and these were included in statistical analysis. P values show the significance of the effect of time for pneuS and pneuE (ANOVA). * shows groups differing significantly from control ($P < 0.05$, Scheffe's *post hoc* test).



Broncho-alveolar lavage of control, unbronchoscoped rabbits contained very few alveolar macrophages containing apoptotic bodies ($2.2 \times 10^4 \pm 1.2 \times 10^4$ and $2.9 \times 10^4 \pm 0.9$ 10^4, mean $\pm$ SEM for the two control groups). In both pneuS and pneuE there was a significant effect of time on the number of these cells ($p = 0.0013$ and $p = 0.003$ respectively). *Post hoc* analysis revealed numbers were significantly raised above control in 24 and 48 hour groups. The trend for the later groups not included in formal analysis was to show even higher levels before returning towards normal, with an eventual maximum of

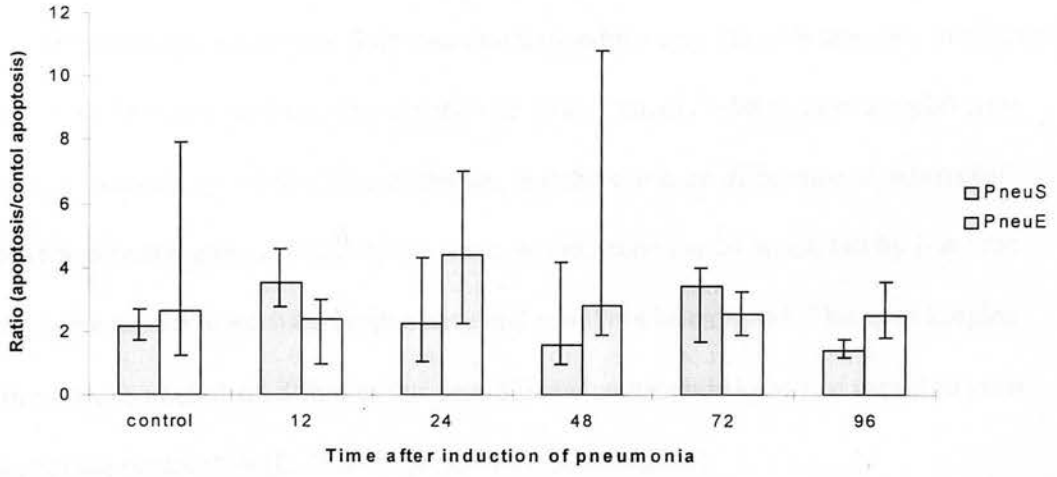
$67.7 \times 10^4 \pm 18.0 \times 10^4$ and $69.6 \times 10^4 \pm 22.5 \times 10^4$ (mean $\pm$ SEM) for pneuS and pneuE respectively, each occurring at 72 hours.

Overall, the results show that there is a decline in total neutrophil numbers form 12 hours onwards, and that the trend is for a later increase in total apoptotic cells. Thus, as neutrophil numbers fall, apoptotic cell numbers rise, and the number of alveolar macrophages containing apoptotic bodies increases. This is consistent with the hypothesis that ageing neutrophils in the lung undergo apoptosis and as they do so they are cleared by alveolar macrophages.

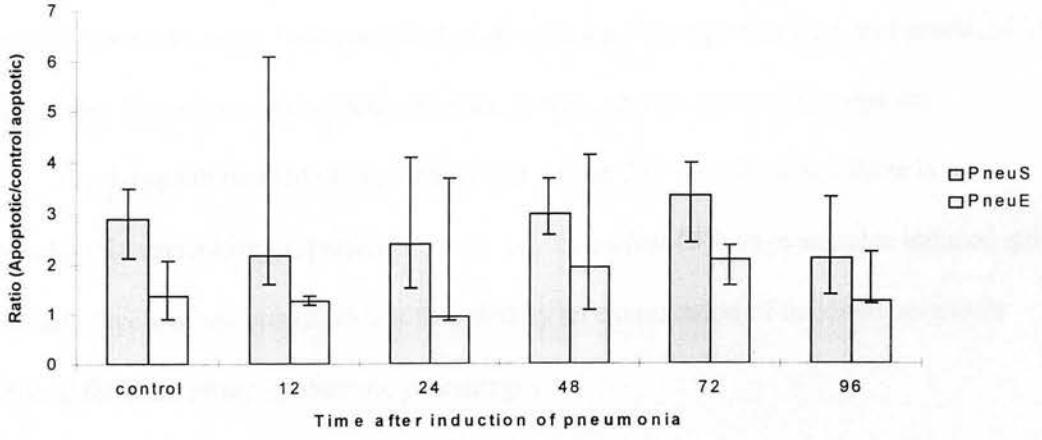
Figure 5-11 a) and b) Effect of broncho-alveolar lavage fluid on rabbit neutrophil apoptosis *in vitro*

The graphs show the effect of culturing rabbit neutrophils with 50 % broncho-alveolar lavage fluid taken at intervals after induction of pneuS or pneuE on the amount of apoptosis in the neutrophils. Data is expressed as the ratio of apoptosis in the presence of test broncho-alveolar lavage fluid and PBS control, showing median apoptotic ratio and interquartile ranges. n = 6 per group. Graph a) shows apoptotic ratio after 5 hours, graph b) shows apoptotic ratio after 10 hours.

a)



b)



Broncho-alveolar lavage supernatants collected from control animals and from animals12, 24, 48, 72 and 96 hours after induction of pneumonia were assessed for their ability to induce apoptosis in rabbit neutrophils *in vitro*, 100 µl being added to 100 µl of

neutrophil suspension. The results are expressed as the ratio of apoptosis induced by the test bronchoalveolar lavage fluid to that produced by a control of PBS. n = 6 for each time point. In absolute terms, the ranges of percent of neutrophils that were apoptotic when culured with PBS for 5, 10 or 20 hours were 0.7-3.3 %, 0.41-15.4 %, and 5.7-21.3 %. For culture with control bronchoalveolar lavage, the ranges were 3.9-11.3 %, 3.06-23.1 % and 4.28-58.9 %. For culture with bronchoalveolar lavage from animals with PneuS the ranges were 2.6-36.5 %, 2.8-53.1% and 5.7 % to 60.7 %. For culture with broncho-alveolar lavage from animals with PneuE the ranges were 0.6-16.1 %, 1.2-33.7 % and 4.9-44.7 %.

Broncho-alveolar lavage fluid was found to induce very variable amounts of apoptosis after 5 and 10 hours. At these time points > 95 % and usually > 98 % of neutrophil were viable as assessed by Trypan Blue exclusion, and there was no difference in neutrophil recovery between groups. Neutrophils were also examined at 20 hours, but by this time many were unable to exclude Trypan Blue and could not be assessed. This is in keeping with previous unpublished studies that found rabbit neutrophils to be less robust *in vitro* than human neutrophils (C. Haslett, personal communication).

Overall, the results at 5 and 10 hours were very variable. However, it was notable that at all time points and in both sorts of pneumonia, as well as unbronchoscoped controls, broncho-alveolar lavage fluid produced neutrophil apoptosis greater than that produced by PBS alone. This means all broncho-alveolar lavage induces neutrophil apoptosis.

The large interquartile ranges show that within this overall picture there is considerable variability. In particular, some broncho-alveolar lavage samples induced quite dramatic levels of apoptosis. This is revealed by an examination of maximal apoptosis induced for each group in absolute percentages.

Table 5.1 Maximum amount of apoptosis produced per group

The table shows the maximum amount of apoptosis produced in rabbit neutrophils by 5 hours culture in the presence of broncho-alveolar lavage fluid from each group

Group (n = 6)	PBS	Control	12 hr	24 hr	48 hr	72 hr	96 hr
Max. apoptosis (%) pneuS	**3.3**	**11.3**	**36.5**	**26.72**	**18.9**	**25.0**	**32.0**
Max. apoptosis (%) pneuE	**3.7**	**7.6**	**5.1**	**15.66**	**16.14**	**7.35**	**9.96**

It is clear that the maximal rates of apoptosis induced by BAL are way in excess of any seen in PBS controls. In *every* experimental group there are cases in which the apoptosis produced is completely outside the control range. Some animals appear to be producing a factor that induces apoptosis in neutrophils, but this effect is highly variable and is not so clearly apparent in pooled data.

In pneuS, there is a highly significant (though weak) relationship between the levels of the three chemokines IL-8, MCP-1 and gro in broncho-alveolar lavage fluid, and neutrophil apoptosis produced by that broncho-alveolar lavage fluid *in vitro* at 5 and (to a slightly lesser extent) at 10 hours. In addition, in pneuS the amount of apoptosis seen *in vitro* at 10 hours correlated significantly (though weakly) with the total apoptotic neutrophils in BAL ($r = 0.49$, $p < 0.01$). The correlation did not quite reach significance at 5 hours ($r = 0.4207$). None of these correlations held true in pneuE.

Table 5.2 Correlation between neutrophil apoptosis induced in culture by broncho-alveolar lavage fluid and broncho-alveolar lavage fluid chemokine levels

The table shows correlation coefficients relating the amount of apoptosis seen in neutrophils cultured in the presence of broncho-alveolar lavage fluid, and the amounts of the chemokines IL-8, MCP-1 and gro measured in the fluid. Data is given as correlation coefficients. * Significant at $p < 0.01$ ** significant at $p < 0.001$
NS = no significant correlation

	IL-8	MCP-1	gro
Neutrophil apoptosis at 5 hr pneuS	**0.5873****	**0.5594****	**0.6215****
Neutrophil apoptosis at 10 hr pneuS	**0.4776***	**0.5635****	**0.5302***
Neutrophil apoptosis at 5 hr pneuE	**NS**	**NS**	**NS**
Neutrophil apoptosis at 10 hr pneuE	**NS**	**NS**	**NS**

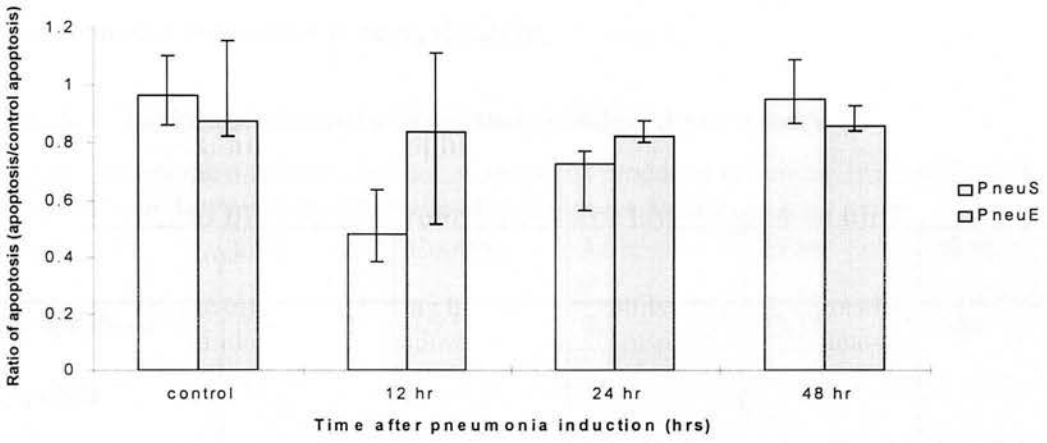
Table 5.3 Ranges of chemokine levels in broncho-alveolar lavage fluid (Minimum, Maximum)

	IL-8 (ng/ml)	MCP-1 (μg/ml)	gro (μg/ml)
Control	0, 0	0, 125.6	0, 0.04
PneuS	0, 2770	0, 400	0, 0.47
PneuE	0, 8207	0, 400	0, 0.81

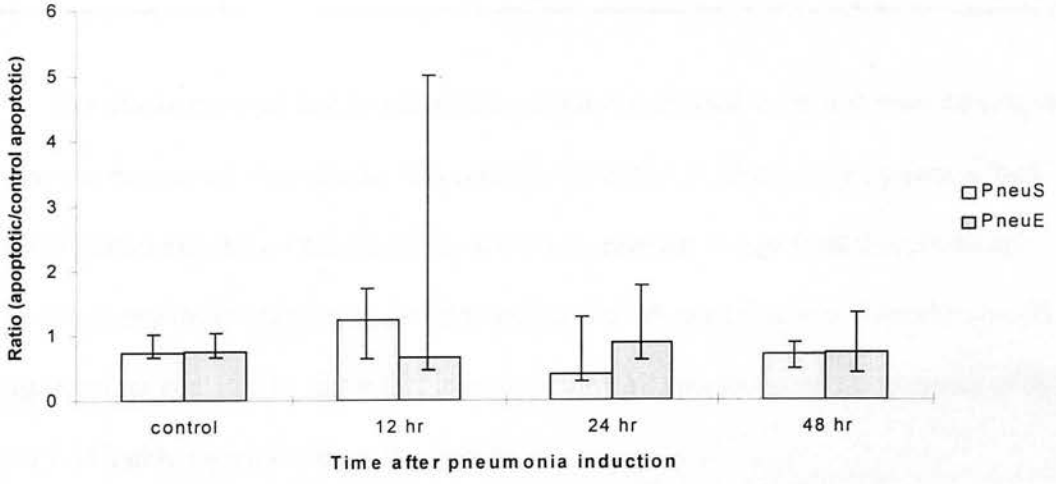
Figure 5-12a) and b) Effect of broncho-alveolar lavage fluid on human neutrophil apoptosis *in vitro*

The graphs show the effect of culturing human neutrophils with 50 % broncho-alveolar lavage fluid taken at intervals after induction of pneuS or pneuE on the amount of apoptosis in the neutrophils. Data is expressed as the ratio of apoptosis in the presence of test broncho-alveolar lavage fluid and PBS control, showing median apoptotic ratio and interquartile ranges. n = 6 per group. Graph a) shows apoptotic ratio after 6 hours, graph b) shows apoptotic ratio after 20 hours.

a)



b)



Broncho-alveolar lavage fluid from a second group of animals was used to assess the ability of rabbit BAL to affect human neutrophil apoptosis. BAL was from

unbronchoscoped controls and 12, 24 and 48 hours after the induction of pneumonia. Viability as assessed by ability to exclude Trypan Blue was >95 % in all cases at 20 hours as well as 6 hours, and in very nearly all cases was > 98%.

Overall, in contrast to when rabbit neutrophils were used there was a tendency towards inhibition of neutrophil apoptosis. However, in pneuS and pneuE in the 6 hour group there were examples of broncho-alveolar lavage fluid that induced apoptosis, particularly BAL from animals 12 hours after the induction of pneumonia. Re-assay of selected samples showed this to be reproducible.

Table 5.4 Maximum amount of apoptosis produced per group

The graph shows the maximum amount of apoptosis produced in human neutrophils by 6 hours culture in the presence of broncho-alveolar lavage fluid from each group

Group (n = 6)	PBS	Control	12 hr	24 hr	48 hr
Max. apoptosis (%) pneuS	**3.5**	**2.93**	**4.7**	**35.12**	**7.36**
Max. apoptosis (%) pneuE	**3.5**	**3.01**	**16.4**	**8.57**	**3.83**

The absolute rate of human neutrophil apoptosis produced in control broncho-alveolar lavage was not outside that seen in PBS controls. However, at all other time points in both types of pneumonia there were examples of broncho-alveolar lavage fluid that produced apoptosis above the range seen in the PBS control. This showed that some broncho-alveolar lavage samples contained a factor that induced neutrophil apoptosis, but the presence of this factor was highly variable even within groups.

At 20 hours, there was very little tendency for broncho-alveolar lavage to either promote or inhibit human neutrophil apoptosis. The only exception to this was in the 12 hour pneuS group where there was a tendency to inhibition, though this did not reach statistical significance.

It is notable that TNF α produces early neutrophil apoptosis and diminished late apoptosis. Pre-incubation of selected BAL samples that produced high 6 hour apoptosis were tested for the ability of anti-TNF antibody to inhibit the pro-apoptotic effect of the samples. No inhibition was demonstrated. In addition, the correlation between apoptosis at 6 hours and 20 hours though relatively weak was positive ($r = 0.418$, $p = 0.040$), unlike the negative relationship seen with TNF α. Further preliminary studies showed incubation of neutrophils with anti-Fas antibody also failed to inhibit the pro-apoptotic activity of the broncho-alveolar lavage fluid. Each of these studies used a control lavage that was not able to induce apoptosis, a lavage from pneuS at 24 hours that showed intermediate ability to induce apoptosis, and a lavage from pneuE at 24 hours that showed great ability to induce apoptosis. Each lavage sample produced consistent effects on apoptosis on each occasion they were studied.

Human neutrophil apoptosis *in vitro* at 6 hours in pneuS produced by broncho-alveolar lavage fluid correlated significantly with total neutrophils recovered from the lavage ($r = 0.597$, $p < 0.01$). No such correlation was seen at 20 hours, or with pneuE. This paralleled the relationship seen with rabbit neutrophils.

5.5 DISCUSSION

5.5.1 Cellular recovery from broncho-alveolar lavage

Neutrophil recovery from selective broncho-alveolar lavage of the right cranial lobe was greatest at 12 hours and declined progressively thereafter in both pneuS and pneuE. In chapter two, broncho-alveolar lavage neutrophil recovery was prolonged in pneuE compared to pneuS. Two differences in technique explain this apparent discrepancy. Firstly, timings of groups in chapter two refer to time of injection of In-wbc. The actual lavages were taken 21 hours later. Thus, '6 hour' and '30 hour' groups were actually lavaged at 27 and 51 hours,

and are more comparable to 24 and 48 hour lavages in this chapter. Examination of neutrophil numbers at these time points reveals a pattern not so dissimilar to that seen in chapter two.

Nevertheless, neutrophil influx to the right lung at 30 hours, assessed using In-wbc, was as high as at 6 hours in pneuE, but not pneuS. The fact that neutrophil recovery fell between these times even in pneuE in studies reported in this chapter is likely to be due to the selective sampling of the cranial and middle lobes in these studies, whereas the entire right lung was sampled in studies for chapter two. From the data presented in chapter two, the ratio of indium influx, (as determined by tissue gamma well counting), between the cranial and middle lobes, and the caudal lobe, in the 6 hour group is 8.24 and 4.24 in pneuS and pneuE respectively, and in the 30 hour group is 3.78 and 1.51. This suggests the inflammation in pneuS is proportionately more confined to the cranial lobe, whereas there is greater spread of influx to the caudal lobe in pneuE. This spread would not be sampled in the current experiment, but would be consistent with the finding that pneuS in clinical disease is more likely to show a lobar pattern whereas gram negative pneumonias more commonly manifest as a more diffuse bronchopneumonia (Coalson, 1995). If the spread of inflammation in pneuE proceeds outwards from the initial focus, late neutrophil influx would be concentrated at the peripheral parts of the lesion, i.e. in the caudal lobe. This would be sampled in the experiments in chapter two, but not in the current experiments, and it accounts for the apparent differences.

As expected, low numbers of morphologically apoptotic neutrophils were recovered. It has been shown that after injection of anti-CD4 antibody sufficient to cause 50 % cell lymphocyte loss after 24 hours, only 2 - 3 % of cells appeared apoptotic on morphological examination (Howie et al, 1993). This is because apoptotic cells are rapidly cleared form tissues (Kerr et al, 1987, Wyllie et al, 1980).

Because of the low numbers present, neutrophils were concentrated from broncho-alveolar lavage by centrifugation on a hetastarch-Percoll gradient. Erythrocytes and neutrophils were deposited at the base of this gradient as a loose pellet. The macrophage rich cell layer on top of the gradient rarely contained any neutrophils, apoptotic or otherwise, so it seems unlikely this gave relative concentration or dilution of apoptotic compared to normal neutrophils. In view of the fact that neutrophil adhesive functions diminish during apoptosis, it is possible that apoptotic cells were selectively lavaged compared to normal neutrophils (Dransfield et al, 1995). However, even though this could give slight overestimation of their numbers, it should still allow demonstration of differences between groups.

Apoptotic neutrophils were recognised by their morphological appearances (Wyllie et al, 1980). The shrunken nature of apoptotic cells, together with nuclear condensation was readily apparent on acridine orange staining. The delicate fine granularity of the chromatin in normal cells was quite different from the uniform, bright staining of chromatin in apoptotic cells. Usually the multilobed nature of the nucleus was retained, but often the lobes appeared to be completely separated. Acridine orange staining was routinely used for quantitation of rabbit neutrophils, as they appeared delicate when apoptotic, often making cytocentrifuge preparations unclear. Nevertheless, examples of clear apoptotic morphology were apparent in the latter. Electron microscopy also confirmed the presence of cells with ultra-structural characteristics of apoptosis.

At least 500 cells were counted per sample with the exception that, in control animals, neutrophil recovery was so low as to preclude this. Usually 100 - 200 cells (but sometimes even fewer) could be counted in the control group. As only a few per cent of neutrophils were apoptotic, this meant a significant degree of variability was inevitable. Despite this, there was a significant effect of time on total apoptotic neutrophil recovery in pneuS, with *post hoc* testing revealing the 24 hour time point to have significantly greater apoptotic

neutrophil recovery than controls. Whilst in pneuE the effect of time fell just short of reaching significance ($p = 0.055$), the overall pattern appeared similar in both types of pneumonia, with a prolonged increase in apoptotic neutrophils. Numbers after 72 hours were similar to those after 12 hours. Thus, the peak of apoptotic cell recovery was broader and delayed compared with that of total neutrophil recovery. Freshly recovered neutrophils hardly ever lost the ability to exclude Trypan Blue dye, meaning they were not undergoing necrosis.

Data relating to alveolar macrophages was more robust due to the larger cell recoveries. They were examined on routine cytocentrifuge preparations stained with Diff-Quik® for the presence of apoptotic bodies. These were recognised as vacuoles containing large basophillically stained inclusions. These were sometimes clearly recognisable as apoptotic neutrophils, although on other occasions, degradation had proceeded to a degree where direct recognition was not possible. It is unlikely that they represented the ingestion of any other cell type more than infrequently. The very large number of neutrophils present initially declined as these cells appeared, and as noted some inclusions could be directly identified as neutrophils. Although epithelial cell damage occurs in pneumococcal pneumonia, it is unlikely they are lost in sufficient numbers to contribute significantly to the number of inclusions seen, or there would be gross destruction of the lung's architecture in a way that is not usually witnessed (Johnston, 1991). In addition, electron microscopy showed examples of alveolar macrophages with vesicles containing partially degraded remnants that were clearly compatible with apoptotic neutrophils. The total number of macrophages conatining such inclusions increased throughout both types of pneumonia.

Only occasional neutrophils recovered from broncho-alveolar lavage were found to be unable to exclude Trypan Blue. Thus, most appeared to be normal or apoptotic, rather than necrotic. Nevertheless, in areas of very intense inflammation, degenerate neutrophils were apparent histologically. Indeed, it would be remarkable if some neutrophils did not suffer a

necrotic death in view of the toxic factors that accumulate at the inflammatroy site. It is virtually impossible to directly compare the number of neutrophils becoming necrotic with those becoming apoptotic because the latter are only transiently present, and the former may be difficult to recognise as more than cellular fragments. The clear demonstration of apoptotic neutrophils at the inflammatory site suggests apoptosis does play a rôle in neutrophil clearance. Furthermore, even the presence of low numbers of apoptotic cells implies the removal of large numbers of cells by this mechanism, as discussed (Howie et al, 1994).

In addition, the temporal sequence of cell recovery follows the pattern expected if neutrophil apoptosis plays a significant rôle in neutrophil clearance, with an initial peak in total neutrophils, followed by a later peak in apoptotic neutrophils, followed by a later peak of alveolar macrophages containing apoptotic bodies,.

It is concluded that apoptosis contributes significantly to neutrophil clearance from the lung during pneumonia.

5.5.2 Comparison of cell recovery data with other studies

As described in the general introduction, a number of historical studies have described neutrophil apoptosis by macrophages during inflammation, without realising the full implications of the process. This includes the description of neutrophils with pyknotic nuclei being taken up by macrophages after 24 to 48 hours of experimental pneumonia (Loosli, 1942b).

1 to 11 % of neutrophils from inflamed synovial fluid were morphologically apoptotic (Savill et al, 1989), and apoptotic neutrophils have also been observed in broncho-alveolar lavage from babies with respiratory distress syndrome (Grigg et al, 1991).

Two abstracts presented to the American Thoracic Society relate to studies of apoptosis of neutrophils *in vivo*. One of these used the TUNEL method to demonstrate

extremely high numbers of apoptotic cells in pneumonia histologically (Kutkoski et al, 1996). As discussed in the general introduction, this method is quite likely to overestimate apoptosis compared to morphological methods, and there must be some doubt to its applicability at inflammatory sites. The study did indeed demonstrate fewer apoptotic neutrophils from broncho-alveolar lavage, these being assessed by morphology. The exact numbers are not given in the abstract.

The other study examined the number of apoptotic cells in broncho-alveolar lavage from patients with ARDS (Matute-Bello et al, 1996). The most surprising fact about this study was that the majority of apoptotic neutrophils were said to be free, with less than 1 % being found within macrophages. The reason for this unique finding is not clear from the abstract. However, between 1 and 3.3 % of neutrophils were found to be apoptotic, of the same order as in the current study.

After instillation of thioglycollate broth into guinea pig peritoneum, there is a neutrophil accumulation that peaks at 24 hours before declining (Yasamato et al, 1995). Macrophages were found to ingest neutrophils, and the maximal proportion of macrophages containing neutrophils was at 48 hours, when 20 % of macrophages showed this phenomenon. Although the experimental model is very different, this is broadly similar to the current study.

A much more similar study was performed to examine apoptotic neutrophil appearance and macrophage uptake after intrapulmonary instillation of LPS in rats. Broncho-alveolar lavage was performed at 6, 18, 24, 48 and 72 hours. Neutrophil recovery was greatest at 18 hours, declining a little by 24 hours. This was commensurate with the current study. The peak in apoptotic cell number occurred at 24 hours. Macrophages showing apoptotic bodies also increased to a maximum at 48 hours, but were still clearly elevated 48 hours later. These timings are in excellent agreement with the current study. The data of Cox et al show very tight peaks of total and apoptotic cell number, with a standard

deviation of roughly 20 % of the mean despite a maximum rate of apoptosis of 3 % of total neutrophil numbers. This is likely to relate to the use of LPS rather than live stimuli. Live stimuli are intrinsically more variable, and early intrapulmonary growth may 'blur' the onset of inflammation so that it varies in different areas, though the use of LPS suffers from the criticism that this is an artificial stimulus. Cox et al found 1 to 3 % of neutrophils were apoptotic overall, whereas this study found 5 to 10 % were apoptotic. Cox et al also found a maximum of 10 % of alveolar macrophages showing evidence of having ingested neutrophils. Interestingly, they found roughly 1.5 free apoptotic neutrophils per alveolar macrophage containing a recognisable apoptotic body, compared to 1 at 24 hours and 2 at 48 hours in the current study, implying a similar removal rate. Overall, the results are in excellent agreement.

5.5.3 *The effect of broncho-alveolar lavage fluid on rabbit neutrophil apoptosis* in vitro

When neutrophils were cultured in a 50:50 mix of broncho-alveolar lavage fluid and culture medium supplemented with serum, a greatly variable rate of apoptosis was observed observed at 5 and 10 hours of culture. At 20 hours of culture there was a significant degree of Trypan Blue positivity, precluding further analysis of this point.

Although the rate of apoptosis was variable, every broncho-alveolar lavage sample produced greater apoptosis than PBS controls, showing that in general, broncho-alveolar lavage was able to induce apoptosis. In addition, at every time point, there were examples of lavages that produced amounts of apoptosis in excess of that produced by any control lavage. Either the expression of the factor in control broncho-alveolar lavage that was able to induce apoptosis was increased, or a further factor was elaborated.

In pneuS, the amount of apoptosis produced by broncho-alveolar lavage fluid correlated with the levels of the chemokines IL-8, gro and MCP-1, the strongest correlation

being with gro. IL-8 has been reported not to have any effect on neutrophil apoptosis *in vitro*, (Colotta et al, 1992), whilst the effect of MCP-1 and gro have not been reported. MCP-1 has generally been found to have no effect on neutrophils. Gro shares receptors with IL-8, and has many overlapping functions, so it would be surprising if it proved to have radically different effects on apoptosis. In addition, as discussed, most pro-inflammatory mediators inhibit rather than enhance apoptosis. It is more likely therefore that this correlation reflects co-expression of an apoptosis promoting substance with the chemokines rather than a direct action.

It is interesting that the relationship between *in vitro* apoptosis and chemokine levels doses not hold true in E. coli pneumonia. This was despite the fact that there was an induction of apoptosis by some examples of broncho-alveolar lavage from pneuE. Broncho-alveolar lavage from an inflammatory site will undoubtedly have a variety of substances capable of modulating apoptosis to some degree, and the final effect will be a balance of these. It seems that the factor that induces apoptosis is not as dominant in pneuE as in pneuS. One possible explanation is the LPS that is likely to be present in lavage from pneuE. This is known to inhibit neutrophil apoptosis *in vitro* (Colotta et al, 1992), and similar effects have been observed *in vivo* (Yamamoto et al, 1993).

The relevance of these findings is suggested by the fact that the amount of apoptosis induced by broncho-alveolar lavage after 10 hours *in vitro* correlates significantly (though weakly) with apoptotic neutrophil recovery *in vivo.* On the other hand, this relationship was not found in pneuE. This finding was confirmed using human neutrophils and a different set of lavages from pneuS and pneuE, when 6 hour neutrophil apoptosis was again found to correlate with *in vivo* neutrophil recovery from pneuS and pneuE. It suggests that the modulation of apoptosis in pneuS is dominated by a factor that induces apoptosis, whereas in pneuE this factor is not able to exert such clear dominance. Differences in conditions,

such as dilutional factors, *in vivo* and *in vitro*, may allow other factors (such as LPS) to dominate.

5.5.4 *The effect of broncho-alveolar lavage fluid on neutrophil apoptosis* in vitro, *and possible mechanisms of broncho-alveolar lavage induction of neutrophil apoptosis*

The effect of rabbit broncho-alveolar lavage fluid was also tested on human neutrophil apoptosis *in vitro.* This was performed as human neutrophils retain the ability to exclude Trypan Blue dye in culture beyond 20 hours. This difference from rabbit cells, which seem much less robust than human cells, has been noted before (C. Haslett, personal communication). In addition, this performs a cross check of the results obtained with rabbit cells in a more fully characterised system (there are no published studies of apoptosis of rabbit neutrophils in culture). Further, it prepares for future studies using blocking antibodies to neutrophil receptors; these reagents are often not available for use with rabbit cells.

Unlike the case with rabbit neutrophils, 6 hour apoptosis of human neutrophils was not enhanced by control broncho-alveolar lavage. However, as in the case of rabbit neutrophils, although there was no consistent pattern throughout pneumonia, there were clear examples of induction of apoptosis by rabbit neutrophils, and this was clearly repeatable for individual lavages. Once again, virtually every time point in both types of pneumonia produced examples of broncho-alveolar lavage fluid capable of induction of apoptosis. The lack of induction with control lavage is most likely to be due to species difference in response. This may be because a single apoptosis-inducing substance is present but stimulates human neutrophils less than rabbit cells so there is little effect at resting concentrations. Alternatively, there may be more than one apoptosis producing substance present, with the predominant substance present in control samples cross-reacting poorly,

whilst that induced during inflammation cross-reacts well. Clearly this at present remains highly speculative.

After 20 hours of neutrophil culture in the presence of broncho-alveolar lavage, there was very little change in the amount of apoptosis. In fact, broncho-alveolar lavage samples collected 12 hours after induction of pneuS tended to inhibit this late neutrophil apoptosis. It is notable that early induction of neutrophil apoptosis with late inhibition is an unusual property possessed by TNFα (Murray et al, In press). However, correlation of apoptosis produced after 6 and at 20 hours culture showed a positive correlation for individual samples, as opposed to the negative one expected if the effect is solely due to TNFα.

The action of Fas-ligand on Fas has also been found to induce apoptosis in neutrophils (Liles et al, 1996). Fas-ligand can be produced by neutrophils. Therefore, at sites of inflammation with elevated neutrophil density, increased Fas-ligand might be expected. This could induce neutrophil apoptosis, providing negative feedback control of tissue neutrophil burden. This merits further investigation, although preliminary experiments have shown the induction of human neutrophil apoptosis by broncho-alveolar lavage fluid is not inhibited by anti-Fas antibody.

A similar negative feedback mechanism is provided by the finding that a variety of proteases that can be produced by the neutrophil cause neutrophil apoptosis (Trevani et al, 1996). This could be useful in sensing an excess liberation of neutrophil enzymes, so that if their concentration is elevated in the internal *milieu*, apoptosis of surrounding neutrophils would be induced. It is possible that tissue proteases in broncho-alveolar lavage fluid mediate the induction of apoptosis in neutrophils. This could also explain the correlation with IL-8 and gro levels, which are known to cause neutrophil degranulation. However, proteases still produce increased apoptosis at 18 hours, rather than the biphasic response seen with human neutrophils.

Although TNFα, Fas-ligand and proteases may contribute to the pro-apoptotic effect of broncho-alveolar lavage, none seem to offer a complete explanation for the phenomenon. These are the only non-pharmacological substances demonstrated to enhance neutrophil apoptosis to date. This raises the possibility that a novel pro-apoptotic factor may be responsible. This could have exciting implications therapeutically, acting to down-regulate inappropriately recruited neutrophils in such diseases as the Adult Respiratory Distress Syndrome, or pulmonary fibrosis, thereby diminishing their capacity to cause damage.

Analogous experiments were performed using synovial fluid from inflamed joints (Bell et al, 1995). Apoptosis of neutrophils was enhanced when cultured with the synovial fluid. This differed from the current study in that apoptosis was enhanced at 24 hours; in fact, earlier effects were not assessed. Interestingly, they also found the effect to be highly variable. Several samples produced no effect, whilst some showed dramatic enhancement (10 to nearly 100 % in one case). They found that GM-CSF was unable to reverse this effect, whereas others have found the stimulatory effects of TNFα can be overcome thus (E. Chilvers, personal communication), suggesting TNFα is not responsible.

Formerly, it has been thought that high concentrations of inflammatory mediators at the inflammatory site lead to prolongation of neutrophil survival there (Haslett, C., 1992). Consideration of the results of the this study and those of Bell et al suggests the situation is more complex. At the inflammatory site the neutrophil is exposed to both pro- and anti-apoptotic signals. It would be very surprising if this ability to modulate apoptosis has evolved accidentally, and this is strong evidence that the apoptosis does indeed have an important part to play in limitation and resolution of the acute inflammatory process.

5.6 Summary

1) Cells recovered from BAL of pneuS and pneuE contained apoptotic neutrophils and alveolar macrophages containing apoptotic, as determined by light and electron

microscopy. Such cells were also seen on light microscopy of tissue sections of pneumonic lung.

2) In both pneuS and pneuE, selective broncho-alveolar lavage of the right cranial lobe recovers cells in the order expected if apoptosis plays a role in neutrophil clearance during pneumonia. Initially, neutrophil levels peak at 12 hours, followed by a broader peak of apoptotic neutrophils extending from 12 to 72 hours. Alveolar macrophages containing apoptotic neutrophils become common from 48 hours.

3) The magnitude and timing of these responses are in good agreement with other studies, and are sufficient to suggest apoptosis plays an important rôle in neutrophil clearance in pneumonia.

4) *In vitro,* rabbit neutrophils cultured in the presence of broncho-alveolar lavage fluid demonstrated induction of apoptosis. This was variable, but in some cases could be quite marked. In pneuS, there was a correlation between induction of apoptosis and chemokine levels, but this was not true in pneuE. In addition, *in vitro* induction of apoptosis correlated with *in vivo* apoptotic neutrophil recovery in pneuS but not pneuE.

5) Using human neutrophils, it was confirmed that some examples of broncho-alveolar lavage from pneuS and pneuE were able to induce early (6 hour) apoptosis. Use of human cells also allowed the effect at 20 hours to be studied. By this time, little effect was apparent, with the exception of an inhibitory action at 6 hours in pneuS. Once again, a correlation between early *in vitro* neutrophil apoptosis induction and *in vivo* apoptotic neutrophil recovery was observed in pneuS but not pneuE, despite the use of different lavage samples.

6) TNFα can produce early stimulation and late inhibition of apoptosis, but there was a positive rather than a negative correlation of 6 and 20 hour apoptosis induced in human cells. Preliminary experiments suggest anti-TNFα antibody is unable to block the effect.

7) Protease and Fas-ligand may also contribute to the pro-apoptotic action of broncho-alveolar lavage fluid and both are likely to be present. However, preliminary data suggests anti-Fas anti-bodies are unable to block the pro-apoptotic effect of broncho-alveolar lavage fluid. Proteases do not produce the biphasic response witnessed with human neutrophils.

8) As the known physiological factors that are pro-apoptotic for neutrophils do not seem to provide adequate explanation for the pro-apoptotic effects seen, this raises the possibility a novel factor or factors may be important. Such a factor could play a crucial part in resolution of acute inflammation.

6. OVERALL SUMMARY

Despite medical advances, pneumonia remains of great importance, both within hospitals and the community at large. Indeed, the emergence of resistant organisms and the increasing incidence of pathological and iatrogenic immunosuppression are likely to increase its importance.

As reviewed, there is abundant evidence that the inflammatory response is carefully regulated during pneumonia. The response depends on the type of initiating stimulus, and its mode of delivery and dose. Variations of any of these parameters may give rise to qualitative and quantitative differences in cellular response. In addition, it is clear the acute inflammatory response is dynamic and evolving rather than static.

Other studies have been noted, showing there are clear differences in neutrophil recruitment in areas supplied by the pulmonary and systemic circulations. A large part of this difference is likely to be due to low pressure, pulsatile blood flow in lungs, coupled with narrow capillary segments that physically inhibit neutrophil passage.

In vitro, many mechanisms have been defined that may contribute to inflammatory control *in vivo*. It is often difficult to predict how these different processes interact to produce the specificity of response noted. This highlights the importance of *in vivo* models that attempt to reintegrate important data gained from important reductionist studies. It emphasises the need for selection of specific models that parallel disease processes as accurately as possible.

Although the neutrophil is essential to host defence, it may cause secondary damage to host tissues. This thesis hypothesised that in CAP, typified by infection with Streptococcus pneumoniae, the remarkably successful restoration of tissue structure and function is due to successful control of tissue neutrophil burden. It hypothesised that the increased lung damage during NP caused by gram negative organisms, such as E. coli, is due to a greater tissue neutrophil burden.

Neutrophil burden is determined by the balance between neutrophil influx and clearance. Relatively few studies have addressed these two separate parameters specifically, and there is little data on how they vary during evolution of disease. A rabbit model of pneumonia allowed these parameters to be examined.

Data presented in chapter two confirmed that there was a prolonged neutrophil influx in pneuS than pneuE, contributing to a greater tissue neutrophil burden. The mechanism underlying this is uncertain, but could relate to greater persistence of viable E. coli than Strep. pneumoniae within the lung.

The contribution of CD18 and IL-8, two molecules thought to be of widespread importance in mediation of inflammation, were then studied. Anti-CD18 antibodies inhibited neutrophil influx in pneuE, and there was no change in this with time. Anti-IL-8 antibodies were rather surprisingly found not to inhibit neutrophil influx in any group studied. The finding of elevated BAL and plasma IL-8 levels after anti-IL-8 antibody raises the possibility that negative feedback loops had been interfered with, although other mechanisms could not be excluded. This emphasises the fact that the *in vivo* consequences of a molecule may not be easily predicted from its *in vitro* effects.

Data in chapter four shows that apoptosis of neutrophils occurs during pneumonia. It also shows that factors are produced during pneumonia that are capable of modulating this. This is potentially important because necrosis of neutrophils could produce significant tissue damage, whereas apoptosis should promote neutrophil removal without concomitant

damage. Quantitation of the exact contribution of apoptosis to neutrophil clearance is difficult, because of the low numbers of apoptotic cells seen at any given time, and no difference in recovery of apoptotic cells between pneuS and pneuE was detected. Nevertheless, the finding that the ability of BAL from pneuS to induce early *in vitro* neutrophil apoptosis correlates with recovery of apoptotic cells *in vitro*, whereas that from pneuE does not, raises the possibility that apoptosis of neutrophils is more tightly controlled in pneuS than in pneuE. This could lead to the decreased lung damage seen in pneuS than pneuE.

Overall, the experiments presented support the hypothesis that there is an increased tissue neutrophil burden in pneuS than in pneuE. Different patterns of neutrophil influx are shown to contribute to this. No changes in CD18 or IL-8 usage were seen to explain modulation of influx. Indeed, blockade of IL-8 unexpectedly produced no inhibition of neutrophil influx. Neutrophil apoptosis occurs in pneumonia, and there is evidence of its active regulation. It may be more tightly regulated in pneuS than pneuE, helping to prevent lung damage in the former.

Further experiments are merited in the future to investigate the reason for differences in influx patterns, and the mechanisms controlling these, and to investigate the factors controlling apoptosis during pneumonia.

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